FOLIC ACID ACTIVITY IN THE LIVER OF SHEEP

1. THE MEASUREMENT OF FOLIC ACID AND CITROVORUM FACTOR

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This paper describes an investigation of some of the factors which affect the release and determination of folic acid and citrovorum factor in the liver of sheep. *Lactobacillus casei* was used to measure total folic acid activity. This organism utilizes pteroylglutamic acid (PGA) and the di- and tri-glutamates, pteroyl-γ-glutamylglutamic acid and pteroyl-γ-glutamyl-γ-glutamylglutamic acid and also citrovorum factor, N^5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid (CF). In equimolecular amounts these four compounds are equally active. *Leuconostoc citrovorum* was used to measure citrovorum factor (Hutchings and Mowat, 1948; Brockman, Roth, Broquist, Hultquist, Smith, Fahrenbach, Cosulich, Parker, Stokstad and Jukes, 1950; Keresztesy and Silverman, 1951; May, Bardos, Barger, Lansford, Ravel, Sutherland and Shive, 1951; Welch and Heinle, 1951).

EXPERIMENTAL.

The two microorganisms were maintained by weekly subculture in the following media.

Medium for *Lactobacillus casei*.

Skim milk powder	50 gm.
Difco-bacto yeast extract	2·5 gm.
Evans hepamino	1·25 gm.
Vitamin B_{12}	5 μg.

H_2O to 500 ml. adjusted to pH 6·8.

Medium for *Leuconostoc citrovorum*.

Difco-bacto beef extract	0·75 gm.
Difco-bacto yeast extract	0·25 gm.
Difco-bacto tryptone	0·62 gm.
Evans peptone	1·25 gm.
Evans hepamino	0·62 gm.
Glucose	2·5 gm.
Na acetate, $3H_2O$	2·5 gm.

H_2O to 250 ml. adjusted to pH 6·8.

Both media were autoclaved for 15 minutes at 15 lb. pressure.

The composition of the two assay media is given in Table 1. The medium for *L. casei* is a modification of that published by Toepfer, Zook, Orr and Richardson (1951), while that for *Leuconostoc citrovorum* is based on the medium used by Steele, Sauberlich, Reynolds and Baumann (1949). The inoculum medium consisted in each case of the assay medium at half strength and containing a commercial liver preparation "Hepamino", Evans, 0·1 p.c. for *L. casei* and 0·25 p.c. for *Leuconostoc*.

After 18 hours' incubation in the inoculum medium the organisms were centrifuged, washed with saline and resuspended in saline to give a standard opacity. One drop of the final suspension was used to inoculate each assay tube.

The assays were carried out in pyrex test tubes 16-17 mm. in internal diameter and 150 mm. in length. Each contained 5 ml. of assay medium and 5 ml. of test solution. Twelve levels of folic acid (PGA) or Ca leucovorin[1] respectively, were used as standards, and extracts were assayed at not less than 3 levels. Four replicates of each level of standard and extract were employed. The tubes were plugged with cotton wool and autoclaved for 10 minutes at 15 lb. pressure. After inoculation and incubation for 72 hours at 37° C.

TABLE 1.

Composition of assay media.

Constituent	for *L. casei* in 1 litre	for *Leuconostoc citrovorum* in 1 litre
	gm.	gm.
HCl hydrolyzed casein	14·4	12
Glucose	48	50
Na acetate $3H_2O$	77	40
	mg.	mg.
DL-tryptophane	240	200
L-cystine	240	200
L-asparagine	720	800
Adenine sulphate	12	10
Guanine HCl	12	10
Xanthine	12	10
Uracil	12	10
p Amino benzoic acid	1	1
Pyridoxine HCl	4	4
Pyridoxal HCl	4	4
Thiamine	0·4	2
Ca pantothenate	0·8	2
Riboflavin	1·0	2
Nicotinic acid	0·8	2
Biotin	20 μg.	10 μg.
Folic acid	—	20 μg.
Thioglycollic acid	80 mg.	—
KH_2PO_4	1·78 gm.	1·78 gm.
$MgSO_4 \cdot 7H_2O$	400 mg.	400 mg.
$FeSO_4 \cdot 7H_2O$	20 mg.	20 mg.
$MnSO_4 \cdot 4H_2O$	264 mg.	79 mg.
Tween 80, 10 p.c.	2 ml.	—

Adjust to pH 6·8 with KOH.

[1] We should like to thank Dr. T. Jukes for a gift of Ca leucovorin.

titratable acidity was measured with N/10 NaOH. Dose response curves were drawn from the titrations of the standard tubes and the concentration of folic acid or leucovorin in the extracts was read from the standard lines. During autoclaving and incubation the tubes were arranged in random order, each set of replicates being randomized separately.

As already stated, the activity for *L. casei* includes that due to citrovorum factor. The Ca leucovorin which was used as a standard in the *Leuconostoc citrovorum* assays is a synthetic dl form of citrovorum factor (Keresztesy and Silverman, 1951). In equimolecular amounts it should, therefore, be 50 p.c. as active for *L. casei* as PGA itself. We measured its activity in two assays in which 7 and 9 concentration levels of Ca leucovorin were used. Weight for weight, it was found to be 38·3 p.c. as active for *L. casei* as folic acid (PGA) (Table 2). The estimates of citrovorum factor made with *Leuconostoc* and using Ca leucovorin as standard have been expressed as "leucovorin". To convert the values to folic acid activity due to citrovorum factor they should be multiplied by 38·3/100 (Tables 3, 4, 5).

TABLE 2.

Activity of Ca leucovorin *for* L. casei.

Assay	Concentration of Ca leucovorin mμg./ml. A	Folic acid activity mμg./ml. B	B/A × 100
1	0·02	0·008	40
	0·04	0·017	42·5
	0·08	0·0315	39·4
	0·20	0·075	37·5
	0·30	0·119	39·7
	0·40	0·152	38
	1·00	0·359	35·9
Mean			39·0
2	0·04	0·0155	38·7
	0·06	0·024	40
	0·08	0·032	40
	0·10	0·0365	36·5
	0·12	0·046	38·3
	0·16	0·059	36·9
	0·40	0·147	36·7
	0·60	0·219	36·5
	0·80	0·282	35·2
Mean			37·6
Mean of assays 1 and 2			38·3

TABLE 3.

Effect of pH and time of incubation on estimate of folic acid and leucovorin.

10 gm. liver in 50 ml. M/5 phosphate: not homogenized.

Liver No. 2.

Initial pH	Incubation time hours	pH after incubation	µg./gm. liver wet wt.		
			Folic acid	Leucovorin	38·3 p.c. leucovorin
with 1 p.c. ascorbic acid					
6·5	4	6·5	3·1	5·1	1·95
	24	5·9	6·1	11·9	4·6
	48	6·2	6·4	12·2	4·7
7·0	0		1·7	1·25	0·48
	4	6·8	4·4	8·3	3·2
	24	6·3	6·7	12·9	5·0
	48	6·4	6·4	12·1	4·6
7·5	4	7·1	4·8	8·4	3·2
	24	6·5	6·6	12·7	4·9
	48	6·5	6·6	12·5	4·8
without ascorbic acid					
7·0	0	6·9	1·18	1·34	0·51
	4		2·6	4·8	1·84

After incubation and before autoclaving pH was measured and adjusted to 6·9-7·0 using a glass electrode.

Liver from retail store.

TABLE 4.

Effect of pH during incubation on estimate of folic acid and leucovorin.

10 gm. liver in 50 ml. M/5 buffer, containing 1 p.c. ascorbic acid: 24 hours' incubation.

Liver	Buffer	Tissue* homogenized after incubation + or −	pH		μg./gm. wet wt.		
			Initial	After incubation	Folic acid	Leucovorin	38·3 p.c. Leucovorin
17	Acetate	+	4·0	4·4	1·70	4·2	1·61
		+	4·5	4·8	1·63	4·3	1·65
		+	5·0	5·2	1·62	3·6	1·38
	Phosphate	+	5·5	5·6	3·1	3·3	1·26
		+	6·0	5·9	4·6	4·75	1·82
		+	6·5	6·1	5·7	8·1	3·1
		+	7·0	6·4	7·4	14·3	5·5
		+	7·5	6·7	7·9	18·0	6·9
		+	8·0	6·8	8·2	19·9	7·6
18	Phosphate	−	7·5	6·8	9·6	12·7	4·9
		−	7·5	6·7	9·8	13·6	5·2
		+	7·5	6·7	10·1	13·2	5·0
		+	7·5	6·8	10·0	13·5	5·2
		−	8·0	6·8	9·6	12·7	4·9
		−	8·0	6·9	9·7	12·2	4·7
		+	8·0	7·0	10·0	12·4	4·7
		+	8·0	6·9	9·8	12·8	4·9
19	Phosphate	−	7·5	6·6	5·9	11·4	4·4
		−	7·5	6·5	5·8	11·6	4·4
		+	7·5	6·6	5·9	11·3	4·3
		+	7·5	6·7	5·8	10·8	4·1
		−	8·0	6·9	5·2	11·0	4·2
		−	8·0	6·8	5·4	11·1	4·2
		+	8·0	6·8	5·9	12·2	4·7
		+	8·0	6·9	5·8	12·5	4·8
20	Phosphate	−	7·5	6·3	7·0	15·8	6·0
		+	7·5	6·5	6·8	14·1	5·4
		−	8·0	6·6	6·3	12·3	4·7
		+	8·0	6·7	6·1	13·3	5·1
Means of 17, 18, 19, 20			7·5 8·0		7·63 7·43	14·37 14·23	

Before autoclaving pH was adjusted to 7·0 with KOH.

* Homogenized after incubation in small stainless steel blender.

Livers from retail stores.

TABLE 5.

Effect of phosphate concentration on the estimate of folic acid and leucovorin.

10 gm. liver in 50 ml. phosphate containing 1 p.c. ascorbic acid, pH 7·5.

Not homogenized.

Concn. of Phosphate in buffer	Incubation time hours	pH after incubation	μg./gm. liver wet wt.		
			Folic acid	Leucovorin	38·3 p.c. Leucovorin
Liver No. 3.					
M/25	24		7·5	11·0	4·2
M/10	24		8·2	14·0	5·4
M/5	24		8·6	13·6	5·2
M/25	48	5·7	6·0	12·0	4·6
M/10	48	6·1	8·7	16·1	6·2
M/5	48	6·4	9·9	15·7	6·0
Liver No. 4.					
M/25	48	5·2	7·8	16·2	6·2
M/10	48	5·8	10·9	28	10·6
M/5	48	6·2	11·4	24	9·1
M/5	48	6·2	8·7	27	10·3
M	48	7·0	8·7	21	8·2
M	48	6·9	11·5	22	8·3

After incubation and before autoclaving pH was measured and adjusted to 6·8 with KOH.
Livers from retail store.

The preparation of liver extracts for assay.

It is known that the greater part of the folic acid and citrovorum factor in the tissues is present in conjugated form as polyglutamates, which cannot be utilized by microorganisms. The liberation of folic acid and citrovorum factor in forms which are available to the microorganisms used for assay can be accomplished by conjugases which are present in liver, kidney, pancreas and other tissues. The tissues, under appropriate conditions, can also convert folic acid to citrovorum factor.

The release of folic acid and citrovorum factor in available form and the conversion of folic acid to citrovorum factor by the enzyme systems in tissues have been studied in several laboratories (Nichol and Welch, 1950; Scheid and Schweigert, 1950; Toepfer, Zook, Orr and Richardson, 1951; Welch and Heinle, 1951; Dietrich, Monson, Gwoh and Elvehjem, 1952; Sauberlich, 1952; Chang,

1953; Doctor and Couch, 1953; Nichol, 1953; Doctor, Couch and Trunnell, 1954; Doctor, Elam, Sparks, Lyman and Couch, 1954; Heisler and Schweigert, 1955, 1956; Reid and Couch, 1955; Doctor, 1956).

In the present series of experiments the splitting of the conjugates in sheep liver has been carried out by means of the conjugases in the liver itself. The tissue was cut into small pieces and 10 gm. samples were covered with a buffer solution, usually 50 ml., 2 ml. toluene were added and the mixture was incubated at 37° C. After incubation the pH was measured and adjusted to neutrality with KOH. Autoclaving for 15 minutes at 15 lb. pressure followed. After cooling, the extracts were made to volume and the insoluble residues were removed by centrifuging or filtering. The extracts were stored at − 20° C. prior to assay. All extracts were tested by both *L. casei* and *Leuconostoc citrovorum* assay procedures.

Determination of optimum reaction and concentration of buffer.

A preliminary experiment indicated that in order to obtain maximal estimates of folic acid and leucovorin the initial pH of the buffer should lie between 7 and 8 and this was confirmed in subsequent tests.

Acetate and phosphate buffers of a molar concentration of 1/5 and containing 1 p.c. ascorbic acid were prepared and 50 ml. were added to 10 gm. samples of liver. The pH of the buffers was 4·0, 4·5, 5·0, 5·5, 6·0, 6·5, 7·0, 7·5 and 8·0. Incubation was for 24 hrs. followed by homogenization in a small stainless steel blender. The pH was then measured and adjusted to 7·0 before autoclaving. In addition, three livers were incubated in phosphate buffers of initial pH 7·5 and 8·0, half the samples being homogenized after incubation. The results of these and other tests are presented in Tables 3 and 4.

An examination of phosphate buffers at pH 7·5 and of varying concentrations, M/25, M/10, M/5 and molar indicated that higher estimates of folic acid and leucovorin were obtained with M/5 or M/10 (Table 5).

As a result of these experiments most subsequent tests were carried out in M/5 phosphate with or without 1 p.c. ascorbic acid and adjusted to an initial pH of 7·5. During incubation the reaction invariably became more acid (Tables 3, 4, 5, 7, 9). Before autoclaving it was adjusted to 7·0.

Effect of time of incubation.

Measurements of folic acid and leucovorin carried out on the fresh tissue without incubation gave low results. Activity increased slowly during autolysis and was always greater after 24 hours than after the shorter periods studied, 2, 4 and 8 hours (Table 6). In three experiments the effect of incubating for 48 hours was examined and in two of them rather higher values were obtained than after 24 hours (Tables 3 and 5).

TABLE 6.

The effect of incubation time on the estimate of folic acid and leucovorin.

M/5 phosphate, pH 7·5: with no incubation, pH 7·0.

Liver No.	24 hours' incubation μg./gm. wet wt.		Folic acid estimate as p.c. of that after 24 hours Incubation time: hrs.				Leucovorin estimate as p.c. of that after 24 hours Incubation time: hrs.			
	Folic acid	Leucovorin*	0	2	4	8	0	2	4	8
Part 1. 1 p.c. ascorbic acid in the buffer.										
7	9·3	21	35	47	54	58	10	29	31	42
5	6·5	13·1	41	22	37		9	17	31	
9	3·1	5·4	23		27	37	6		18	25
6	0·97	1·69	18	22	30	47	5	5	11	31
8A	0·64	0·64	12		26	47	2		14	31
B	0·55	0·62	24		45	56	5		24	47
Part 2. No ascorbic acid.										
7	8·2	13·0	17	55	53	67	11	67	62	78
5	4·3	5·7	26	39	43		22	8	34	
9	2·4	4·5	16		26	37	4		16	22
6	0·83	0·97	7	11	20	33	4	2	5	10
8A	0·32	0·40	7		23	51	2		15	28
B	0·39	0·56	9		38	56	2		19	32

After incubation and before autoclaving pH was measured and adjusted to 7·0.
Liver Nos. 5, 7 from retail store.
Liver Nos. 6, 8A from laboratory, cut up immediately after slaughter.
Liver Nos. 8B and 9 from laboratory, chilled in refrigerator overnight before cutting up.
Liver Nos. 5 and 6 homogenized after incubation.
Liver Nos. 7, 8, 9 not homogenized.
* The Ca leucovorin which was used as a standard was ≡ 38·3 p.c. of its wt. of folic acid for *L. casei*.

A comparison of estimates made after 24 hours' incubation with those carried out on the unautolyzed tissue showed that the increase in leucovorin was always greater than the increase in folic acid. When the buffer contained 1 p.c. ascorbic acid leucovorin usually increased 10 to 20 times while the corresponding increases in folic acid were 2 to 6 times. The proportion of the total folic acid activity attributable to citrovorum factor varied from 8 to 28 p.c. with no incubation (mean 19 p.c.) and increased after 24 hours to 40 to 85 p.c. (mean 68 p.c.) (Tables 3 and 6).

The role of ascorbic acid

Ascorbic acid has been shown to increase the estimate of citrovorum factor in autolyzing horse liver (Chang, 1953). The CF produced on incubation of

rat liver slices and chick liver homogenates was increased in the presence of ascorbic acid and the conversion of added PGA to CF by rat liver slices, by chick liver homogenates and by cell-free extracts of *L. casei* was also markedly increased (Nichol *et al.*, 1950; Nichol, 1953; Doctor *et al.*, 1954; Reid *et al.*, 1955; Heisler *et al.*, 1955; Doctor, 1956).

Keresztesy and Silverman (1953) fractionated a soluble enzyme preparation from horse liver and obtained a fraction which cleaved citrovorum factor in the presence of l-glutamic acid with the production of an arylamine. Futterman (1956) and Futterman and Silverman (1957) reported that chick liver homogenates destroyed CF by converting it to a labile reduced derivative which subsequently, on exposure to air, split non-enzymically to stoichiometric amounts of p-aminobenzoylglutamic acid and to a pteridine which was not identified. Finally, Silverman, Keresztesy, Koval and Gardiner (1957) used an enzyme preparation purified from hog liver to study the reactions involved. In the presence of a large excess of l-glutamate CF was completely converted to a labile intermediate identified as tetrahydrofolic acid (THFA), the other product of the reaction being N-formyl-l-glutamic acid. Ascorbic acid protected THFA from destruction and in its presence the reaction was shown to be reversible. The reaction rate both ways was reduced by increasing the concentration of ascorbic acid.

Dinning, Sime, Work, Allen and Day (1957) studied the metabolic conversion of folic acid and citrovorum factor to a diazotizable amine. On incubation with liver slices or homogenates CF was cleaved and the amine was produced. Folic acid yielded an amine when incubated with liver slices, but homogenates were ineffective apparently because they failed to convert folic acid to CF. Ascorbic acid strongly inhibited the cleavage of CF in rat and rabbit livers. The cleavage reaction was shown with livers of pigeons, rats, dogs, mice and rabbits but other tissues of the rabbit were inert.

Nichol, Anton and Zakrzewski (1955) described the production of a precursor of citrovorum factor on incubating pigeon liver preparations with PGA in the presence of ascorbate. Subsequent heating converted the precursor to CF. They considered it to be a derivative of PGA at the tetrahydro level of reduction. A heat labile precursor of CF in urine was identified as N^{5-10}-formyl-THFA (anhydrocitrovorum factor, anhydroleucovorin). When autoclaved in the presence of ascorbate it was converted to CF (Silverman, Ebaugh and Gardiner, 1956).

Tetrahydropteroylglutamic acid (THFA) has been shown to play an important part in the transfer of one carbon fragments. Both the N^5 and N^{10} positions on the molecule are involved and the reactive compounds are N^{5-10}-formyl and N^{5-10}-hydroxymethyl PGA (Blakley, 1954; Jaenicke, 1956; Kisliuk, 1956, 1957; Hatefi, Osborn, Day and Huennekens, 1957; Osborn and Huennekens, 1957; Osborn, Vercamer, Talbert and Huennekens, 1957; Peters and Greenberg, 1957).

We have studied the effect on the estimation of folic acid and citrovorum factor of adding 1 p.c. ascorbic acid to the phosphate buffer in which the liver samples were incubated. The final extracts, after 24 hours' incubation, autoclaving for 15 minutes at 120° C. and filtering, were found to contain from one-third to two-thirds of the ascorbic acid present at the beginning, as measured by indophenol titration.

In the presence of ascorbic acid the estimates of folic acid and leucovorin were always increased. The values may be expressed as a percentage of those obtained without ascorbic acid. After 24 (or 48) hours' incubation followed by autoclaving the figures for 10 livers varied from 111 to 168 (mean 132) for folic acid, and from 120 to 230 (mean 164) for leucovorin (Tables 6, 7, 10). When the liver samples in phosphate buffer were autoclaved without incubation the mean figures for 5 livers were 261 and 162 for folic acid and leucovorin respectively (Table 6).

It has already been reported that with ascorbic acid present the proportion of the total folic acid activity after 24 hours' incubation which was attributable to citrovorum factor varied from 40 to 85 p.c., mean 68 p.c. When no ascorbic acid was present the figures were 45 to 71, mean 56.

The effect of homogenizing the tissue before incubation.

The results of these tests are presented in Table 7. The liver was cut up as usual and 10 gm. samples were covered with phosphate buffer. Those to be homogenized were transferred either to a Waring blendor or to a small steel blender of one-third the capacity. With the Waring blendor extra liquid was needed to cover the blades and 80 ml. water or buffer were used making a total volume of 130 ml. as compared with 50 ml. buffer when the tissue was not homogenized. Extra liquid was not required when the small blender was used, but in this case the mixture set to a viscous gel during incubation. The estimates of both folic acid and leucovorin were reduced (Liver No. 13).

The increase in acidity which occurred during incubation of the tissue was reduced by homogenization (Liver Nos. 13, 21, 22, 25).

The standard procedure was compared with several modifications involving maceration in the Waring blendor. With one group of livers, instead of the 50 ml. phosphate at pH 7·5 the following solutions were used: (1) 50 ml. buffer and 80 ml. H_2O (Liver Nos. 21 and 22); (2) 130 ml. buffer at pH 7·5 (Liver Nos. 23 and 24); (3) 130 ml. buffer at pH 7·0 (Liver Nos. 26 and 28). With Liver No. 25 six treatments were compared, i.e. using 50 ml. buffer at pH 7·0 and 7·5, 130 ml. buffer at pH 7·0 and 7·5 and 130 ml. buffer at pH 7·0 and 7·5 with homogenization in the blendor. This last test showed that when the volume of buffer, either at pH 7·0 or 7·5, was increased the estimates of folic acid and leucovorin were decreased. Homogenization reduced the values still further. In one case only, Liver No. 23, was there a definite increase following homogenization.

TABLE 7.

The effect of homogenizing the tissue before incubation on the estimate of folic acid and leucovorin.

10 gm. liver in M/5 phosphate.

Part 1. 1 p.c. ascorbic acid in the buffer.

Liver No.	Homogenization	Incubation		Buffer pH	pH after incubn.	μg./gm. wet wt.	
		Time hrs.	Under Air or N_2			Folic Acid	Leucovorin†
21	— 50 ml. buffer	48	Air	7·5	6·1 6·0	7·6 7·2	17·8 17·0
	+ W.B.* 50 ml. buffer 80 ml. H_2O	48	Air	7·5	7·2 7·1	7·3 7·2	14·2 15·0
22	— 50 ml. buffer	48	Air	7·5	6·0 5·9	0·95 1·06	1·30 1·77
	+ W.B.* 50 ml. buffer 80 ml. H_2O	48	Air	7·5	6·4 6·1	0·85 1·01	1·75 1·94
13	— 50 ml. buffer	24	Air N_2	7·5	7·0 6·9	1·60 2·0	2·9 3·7
	+ small steel blender 50 ml. buffer	24	Air N_2	7·5	7·3 7·5	1·40 1·39	2·0 2·0
23	— 50 ml. buffer	24	Air	7·5	6·3 6·3	5·2 5·2	13·2 13·2
	+ W.B.* 130 ml. buffer	24	Air	7·5	6·8 6·8	6·7 6·3	17·3 17·5
24	— 50 ml. buffer	24	Air	7·5	6·9 7·0	5·1 4·5	14·0 13·5
		24	N_2	7·5	7·0 7·0	4·7 4·7	14·0 14·4
	+ W.B.* 130 ml. buffer	24	Air	7·5	7·4 7·4	4·2 4·35	7·9 9·4
		24	N_2	7·5	7·4 7·4	4·1 4·2	9·0 9·3
26	— 50 ml. buffer	24	Air	7·5	6·1	6·9	12·5
	+ W.B.* 130 ml. buffer	24	Air	7·0	6·9	5·8	11·9
28	— 50 ml. buffer	24	Air	7·5	6·5 6·6	4·6 4·5	8·9 8·2
	+ W.B.* 130 ml. buffer	24	Air	7·0	6·9 6·9	4·0 4·3	7·2 7·3

TABLE 7—*continued.*

Liver No.	Homogenization	Incubation		Buffer pH	pH after incubn.	μg./gm. wet wt.	
		Time hrs.	Under Air or N_2			Folic Acid	Leucovorin†
25	— 50 ml. buffer	24	Air	7·0	6·3 6·3	7·2 7·1	18·6 16·6
	— 130 ml. buffer	24	Air	7·0	6·7 6·7	6·6 7·1	14·9 15·9
	+ W.B.* 130 ml. buffer	24	Air	7·0	7·0 6·9	6·0 6·3	14·0 14·7
	— 50 ml. buffer	24	Air	7·5	6·6 6·6	7·3 6·6	17·7 15·3
	— 130 ml. buffer	24	Air	7·5	7·2 7·1	6·3 6·9	12·9 14·8
	+ W.B.* 130 ml. buffer	24	Air	7·5	7·3 7·3	5·9 5·5	10·8 10·5
	Part 2. No ascorbic acid in the buffer.						
21	— 50 ml. buffer	48	Air	7·5	6·3 6·3	7·0 6·3	10·4 9·0
	+ W.B.* 50 ml. buffer + 80 ml. H_2O	48	Air	7·5	6·5 6·5	4·7 4·6	2·8 3·5
22	— 50 ml. buffer	48	Air	7·5	6·2 6·1	0·68 0·63	0·70 0·64
	+ W.B.* 50 ml. buffer + 80 ml. H_2O	48	Air	7·5	6·3 6·3	0·97 0·56	1·47 0·62

After incubation and before autoclaving measure and adjust pH to 7·0.
W.B.* Waring blendor used to homogenize the tissue.
† The Ca leucovorin which was used as a standard was ≡ 38·3 p.c. of its wt. of folic acid for *L. casei*.

Microscopic examination of the brei after maceration in the Waring blendor showed that a considerable proportion of the liver cells had been ruptured.[2] Sauberlich (1952) reported that homogenates of rat liver and kidney were "virtually incapable" of carrying out the conversion of folic acid to citrovorum factor successfully achieved with slices. He seems not to have used ascorbic acid. Most of our experiments were carried out with ascorbic acid in the buffer (Table 7, part 1), but with two livers tests without ascorbic acid were also included (Table 7, part 2). With one of these, Liver No. 21, there was a much

[2] We should like to thank Mr. F. Fraser for making this examination.

more marked effect of homogenization on leucovorin than on total folic acid activity. Nichol and Welch (1950) reported that rat liver homogenates in contrast to slices formed very little citrovorum factor from added PGA and Dinning *et al.* (1957) also concluded that rabbit liver homogenates were unable to carry out the PGA to CF conversion.

The effect of homogenizing the tissue after incubation. Several tests have been carried out to determine whether homogenizing the tissue after incubation and before autoclaving would liberate larger amounts of folic acid and CF. The Waring blendor and the small steel blender were used. The differences between the treatments were small and statistical examination showed them to be not significant (Table 8).

TABLE 8.

The effect of homogenizing the tissue after incubation on the estimate of folic acid and leucovorin. M/5 phosphate with 1 p.c. ascorbic acid, pH 7·5: 24 hours' incubation.

Liver No.	Method of Homogenizing	μg./gm. wet wt.			
		Folic acid		Leucovorin†	
		Not Homogd.	Homogd.	Not Homogd.	Homogd.
8	Waring blendor with 80 ml. added H_2O	0·64 0·55	0·64 0·66	0·64 0·62	0·78 0·71
9	Waring blendor with 80 ml. added H_2O	3·1	3·2	5·4	5·8
10 (48 hrs.)	Waring blendor with 80 ml. added H_2O	5·5 5·2	5·4 5·4	12·0 12·0	11·8 12·1
35	Waring blendor with 80 ml. added H_2O	1·24 1·41	1·33 1·35	2·4 2·7	2·3 2·4
36	Waring blendor with 80 ml. added H_2O	3·9 4·2	5·4 4·4	6·1 7·0	8·3 6·5
13	Small steel blender	1·60	1·86	2·9	3·5
16	Small steel blender	5·4	4·1	7·4	6·3
18	Small steel blender	9·6 9·8	10·1 10·0	12·7 13·6	13·2 13·5
19	Small steel blender	5·9 5·8	5·9 5·8	11·4 11·6	11·3 10·8
20	Small steel blender	7·0	6·8	15·8	14·1

Mean difference between treatments—
Folic acid: Homogenized—not homogenized = +·0938, not significant.
Leucovorin: Homogenized—not homogenized = −·0544, not significant.

† The Ca leucovorin which was used as a standard was ≡ 38·3 p.c. of its wt. of folic acid for *L. casei*.

The effect of autolysis under nitrogen. Chang (1953) reported that incubation under nitrogen did not increase the estimate of citrovorum factor in autolyzing horse liver when ascorbic acid was present. Nichol (1953), however, working with chick liver homogenates found that nitrogen had an additional positive effect on the conversion of added PGA to CF. Doctor *et al.* (1954) also reported an effect of nitrogen in the presence of ascorbic acid both on the measurement of CF in liver and on the conversion of added PGA to CF.

TABLE 9.

The effect of incubation under air or under nitrogen on the estimate of folic acid and leucovorin. 10 gm. liver in 50 ml. M/5 phosphate containing 1 p.c. ascorbic acid, pH 7·5: 24 hours' incubation.

Liver No.	Incubated under	pH after incubation	μg./gm. wet wt.	
			Folic acid	Leucovorin†
9	Air	6·3	3·1	5·4
	N_2	6·2	3·2	5·5
		6·2	3·0	5·5
10	Air		5·4*	11·8*
			5·4*	12·1*
			5·5	12·0
48 hours' incubation			5·2	12·0
	N_2		5·6*	11·7*
			5·8*	12·2*
			5·3	11·8
			5·6	12·4
11	Air	6·5	5·5	12·9
	N_2	6·5	5·8	12·9
12*	Air	6·8	0·78	1·05
	N_2	6·8	0·71	1·17
14*	Air	6·6	4·7	10·1
	N_2	6·8	4·4	7·7
15	Air	6·7	1·33	2·5
	N_2	6·8	1·17	2·3
29	Air	6·4	1·86	2·4
		6·4	1·81	2·6
	N_2	6·7	1·62	1·66
		6·6	1·82	1·52
30	Air	6·0	7·1	16·8
		6·0	6·9	17·1
	N_2	6·2	7·0	16·5

After incubation and before autoclaving pH was adjusted to 7·0.

* Homogenized after incubation, the rest were not homogenized.

† The Ca leucovorin which was used as a standard was ≡ 38·3 p.c. of its wt. of folic acid for *L. casei*.

TABLE 10.

Recovery of folic acid added before incubation.

10 gm. liver, 50 ml. M/5 phosphate, pH 7·5. No homogenization.

Liver No.	1 p.c. A.A.* in buffer + or −	Incubn. under Air or N_2	Folic acid				
			Found in 10 gm. μg. A	Added μg. B	Estimated expectation μg. A+B	Found μg. F	F/A+B × 100
29	+	Air	18·6 18·1			23·8 26·2	
			Mean 18·35	9·3	27·65	Mean 25·0	90
	+	Air	18·6 18·1			55·5 56·9	
			Mean 18·35	58·7	77·05	Mean 56·2	73
	+	N_2	16·2 18·2			20·5 22·6	
			Mean 17·2	9·3	26·5	Mean 21·55	81
	+	N_2	16·2 18·2			58·0 55·5	
			Mean 17·2	58·7	75·9	Mean 56·7	75
30	+	Air	71 69			115·5 114	
			Mean 70	58·7	128·7	Mean 114·7	89
	+	N_2	71 69			107 111	
			Mean 70	58·7	128·7	Mean 109	85
31	+	Air	44 44			91·0 90·7	
			Mean 44	58·7	102·7	Mean 90·85	88
	−	Air	37 40				
			Mean 38·5	58·7	97·2	87·4	90
32	+	Air	11·4 11·6			22·7 22·3	
			Mean 11·5	14·7	26·2	Mean 22·5	86
	+	Air	11·4 11·6			59·0 61·9	
			Mean 11·5	58·7	70·2	Mean 60·45	86

TABLE 10—*continued.*

Liver No.	1 p.c. A.A.* in buffer + or −	Incubn. under Air or N_2	Folic acid				
			Found in 10 gm. μg. A	Added μg. B	Estimated expectation μg. A+B	Found μg. F	F/A+B × 100
32	−	Air	8·9 7·3				
			Mean 8·1	58·7	66·8	59·4	89
33	+	Air	51 54 54 49 52 53			102 94 109 101 107 100	
			Mean 52·2	58·7	110·9	Mean 102·2	92
	−	Air	41 46			82·8 89·7	
			Mean 43·5	58·7	102·2	Mean 86·2	84

* Ascorbic acid.

In tests made in this laboratory the autolysis of some of the liver samples took place in tightly stoppered pressure flasks in which the air was replaced by nitrogen. The rest of the procedure, measuring and adjusting the pH and autoclaving, was carried out in air. No attempt was made to determine if nitrogen would have an effect without ascorbic acid. The results are presented in Table 9. It is obvious that there is no advantage in using nitrogen during incubation.

Recovery of folic acid added before incubation. Known amounts of folic acid in 2 ml. buffer were added before incubation to flasks containing liver and buffer and the subsequent procedures were carried out in the usual way. Folic acid recoveries were estimated by expressing the total folic acid found as a percentage of the total folic acid expected (Table 10). In these experiments 10 gm. liver were incubated under 50 ml. M/5 phosphate, pH 7·5, for 24 hours. The tissues were not homogenized. With two livers the effect of incubating under nitrogen, and in three others the effect of omitting the 1 p.c. ascorbic acid were examined. These alternatives had little, if any, effect on the percentage recovery of folic acid and all the results may be considered together. The recoveries were found to vary from 73 to 92 p.c. with a mean of 85 p.c. The method of calculation presents the recoveries in the most favourable light. An alternative method would give lower results, i.e. by expressing the difference between the folic acid found in the flasks with and without added folic acid

as a percentage of the folic acid added. Calculated in this way the figures 73 and 92 above become 65 and 85.

The mean loss of folic acid was thus about 15 or 23 p.c. according to the method of calculation. On the other hand, folic acid added to the extracts before assay was estimated quantitatively. In view of these findings, it seems unlikely that the whole of the folic acid activity present in the liver in free and combined form was determined. The rise in activity which took place during incubation probably masked a concurrent destruction. In our present state of knowledge it seems reasonable to suppose that some cleavage of citrovorum factor occurred with the production of p-aminobenzoylglutamic acid. Ascorbic acid has been shown to inhibit this cleavage and to confer a measure of protection on CF, but it has not been claimed to prevent the reaction altogether.

SUMMARY.

A study has been made of some of the factors which affect the determination of folic acid and citrovorum factor in the liver of sheep.

The measurements were made by means of microbiological assays with *Lactobacillus casei* and *Leuconostoc citrovorum* after autolysis of the tissue, usually in phosphate buffer.

The effects of varying the pH and concentration of buffer and the period of autolysis were examined. Homogenization of the tissue before or after incubation in buffer was compared with cutting it into small pieces by hand. The actions of ascorbic acid and of nitrogen were also investigated.

The procedure which was finally adopted was tested by measuring the recovery of folic acid added to the liver at the beginning of autolysis.

Quantitative recoveries were not obtained, the loss being about 15 p.c. or 23 p.c. according to the method of calculation.

It is suggested that some cleavage of citrovorum factor may have taken place.

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REFERENCES.

Blakley, R. L. (1954): Biochem. J., 58, p. 448.

Brockman, J. A., Roth, B., Broquist, H. P., Hultquist, M. E., Smith, J. M., Fahrenbach, M. J., Cosulich, D. B., Parker, R. P., Stokstad, E. L. R., and Jukes, T. H. (1950): J. Amer. chem. Soc., 72, p. 4325.

Chang, S. C. (1953): J. biol. Chem., 200, p. 827.

Dietrich, L. S., Monson, W. J., Gwoh, H., and Elvehjem, C. A. (1952): *Ibid.*, 194, p. 549.

Dinning, J S., Sime, J. T., Work, P. S., Allen, B., and Day, P. L. (1957): Arch. Biochem. and Biophys., 66, p. 114.

Doctor, V. M. (1956): J. biol. Chem., 222, p. 959.

Doctor, V. M., and Couch, J. R. (1953): *Ibid.*, 200, p. 223.

Doctor, V. M., Couch, J. R., and Trunnell, J. B. (1954): Proc. Soc. exp. Biol. and Med., 87, p. 228.
Doctor, V. M., Elam, J. F., Sparks, P., Lyman, C. M., and Couch, J. R. (1954): Arch. Biochem. and Biophys., 48, p. 249.
Futterman, S. (1956): Fed. Proc., 15, p. 258.
Futterman, S. (1957): J. biol. Chem., 228, p. 1031.
Futterman, S., and Silverman, M. (1957): *Ibid.*, 224, p. 31.
Hatefi, Y., Osborn, M. J., Day, L. D., and Huennekens, F. M. (1957): *Ibid.*, 227, p. 637.
Heisler, C. R., and Schweigert, B. S. (1955): Fed. Proc., 14, p. 436.
Heisler, C. R., and Schweigert, B. S. (1956): *Ibid.*, 15, p. 272.
Hendlin, D., Koditschek, L. K., and Soars, M. H. (1953): J. Bact., 65, p. 466.
Hutchings, B. L., and Mowat, J. H. (1948): Vitamins and Hormones, 6, p. 1.
Jaenicke, L. (1956): Fed. Proc., 15, p. 281.
Keresztesy, J. C., and Silverman, M. (1951): J. Amer. chem. Soc., 73, p. 5510.
Keresztesy, J. C., and Silverman, M. (1953): *Ibid.*, 75, p. 1512.
Kisliuk, R. L. (1956): Fed. Proc., 15, p. 289.
Kisliuk, R. L. (1957): J. biol. Chem., 227, p. 805.
May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L., and Shive, W. (1951): J. Amer. chem. Soc., 73, p. 3067.
Nichol, C. A. (1953): J. biol. Chem., 204, p. 469.
Nichol, C. A., Anton, A. H., and Zakrzewski, S. F. (1955): Science, 121, p. 275.
Nichol, C. A., and Welch, A. D. (1950): Proc. Soc. exp. Biol. and Med., 74, pp. 52 and 403.
Osborn, M. J., Freeman, M., and Huennekens, F. M. (1958): *Ibid.*, 97, p. 429.
Osborn, M. J., and Huennekens, F. M. (1957): Biochem. et Biophys. Acta., 26, p. 646.
Osborn, M. J., Vercamer, E. N., Talbert, P. T., and Huennekens, F. M. (1957): J. Amer. chem. Soc., 79, p. 6565.
Peters, J. M., and Greenberg, D. M. (1957): J. biol. Chem., 226, p. 329.
Reid, B. L., and Couch, J. R. (1955): Arch. Biochem. and Biophys., 56, p. 388.
Sauberlich, H. E. (1949): Fed. Proc., 8, p. 247.
Sauberlich, H. E. (1949): Arch. Biochem., 24, p. 224.
Sauberlich, H. E. (1952): J. biol. Chem., 195, p. 337.
Scheid, H. E., and Schweigert, B. S. (1950): *Ibid.*, 185, p. 1.
Silverman, M., Ebaugh, F. G., and Gardiner, R. C. (1956): *Ibid.*, 223, p. 259.
Silverman, M., Keresztesy, J. C., Koval, G. J., and Gardiner, R. C. (1957): *Ibid.*, 226, p. 83.
Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A. (1949): *Ibid.*, 177, p. 533.
Toepfer, E. W., Zook, E. G., Orr, M. L., and Richardson, L. R. (1951): U.S.D.A. Agriculture Handbook, No. 29, p. 3.
Welch, A. D., and Heinle, R. W. (1951): Pharmacol. Rev., 3, p. 345.

FOLIC ACID ACTIVITY IN THE LIVER OF SHEEP

2. THE EFFECT OF "4-AMINO"-PTEROYLGLUTAMIC ACID *IN VITRO*

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The synthesis of "4-amino"-pteroylglutamic acid (aminopterin) was first carried out in 1947 (Seeger, Smith and Hultquist, 1947; Seeger, Cosulich, Smith and Hultquist, 1949). It is a powerful folic acid antagonist.

The inhibitory effect of aminopterin on the growth of *Leuconostoc citrovorum* was reversed by anti-pernicious anaemia preparations and by liver extracts (Sauberlich, 1949). Broquist, Stokstad and Jukes (1950) reported that the inhibition was reversed competitively by citrovorum factor. Aminopterin inhibited the conversion of pteroylglutamic acid (PGA) to citrovorum factor (CF) by *L. arabinosus, S. faecalis, L. casei* and *Leuconostoc citrovorum*. The inhibition indices for pteroylglutamic acid "compared favorably" with those for citrovorum factor when *L. casei* and *S. faecalis* were used as test organisms (Hendlin, Koditschek and Soars, 1953).

Nichol and Welch (1950), working with rat liver slices, and Reid and Couch (1956) with chick liver homogenates, found that aminopterin blocked the conversion of added PGA to CF. The effect on the production of CF when PGA was not added was very small. Nichol and Welch concluded that CF may be formed from precursors in the tissues by reactions which are not inhibited by aminopterin. Nichol, Anton and Zakrzewski (1955) found that aminopterin blocked the formation by pigeon liver of a precursor of CF from PGA, but was without effect on the subsequent conversion to CF by heating.

The production by rabbit liver slices of a diazotizable amine from PGA was prevented by aminopterin but the inhibitor was without effect on the cleavage of CF to give the amine. Hence it was concluded that the inhibition occurred in the conversion of PGA to CF (Dinning, Sime, Work, Allen and Day, 1957).

Futterman and Silverman (1957) found that aminopterin blocked the reduction of folic acid which preceded its cleavage to an amine, and Futterman (1957)

found that the reduction of both PGA and dihydro-PGA was blocked. Extremely low concentrations of aminopterin were effective in preventing the reduction of dihydro-PGA to tetrahydro-PGA by a purified reductase from chick liver (Osborn, Freeman and Huennekens, 1958).

Aminopterin interferes with the utilization of citrovorum factor as well as with its production from PGA (Jukes, 1953).

A study has been made in this laboratory of the conditions which affect the determination of folic acid and citrovorum factor in the liver of sheep (Dawbarn, Hine and Smith, 1958). It was thought that the presence of aminopterin during autolysis of the liver tissue might inhibit the conversion of folic acid to citrovorum factor. If this were so it should be possible to distinguish between CF produced from folic acid and that derived from other precursors.

EXPERIMENTAL.

It has been shown that the Ca leucovorin used as a standard in the assays with *Leuconostoc citrovorum* was 38·3 p.c. as active as folic acid for the growth of *L. casei* (Dawbarn *et al.*, 1958). It follows that folic acid, if wholly converted to CF should be more active than the standard leucovorin by a factor of 100/38·3.

TABLE 1.

Inhibition index for aminopterin.

Aminopterin	Leuconostoc citrovorum				Lactobacillus casei		
	Ca leucovorin mμg./ml.[3]	Inhibition index	Ca leucovorin[4] $\times \frac{38\cdot3}{100}$	Inhibition index	Folic acid activity mμg./ml.[3] as folic acid	Folic acid activity mμg./ml.[3] as Ca leucovorin	Inhibition index
L[1]	0·15	357	0·057	930	0·02		0·35
	0·30	257	0·115	670	0·04		0·30
	0·30	250	0·115	650	0·08		0·31
						0·019	0·29
						0·038	0·20
						0·076	0·14
J[2]					0·03		2·0
						0·038	1·3

[1] A commercial product.
[2] A crude preparation.
[3] Concentration in the final solution after adding the medium.
[4] The Ca leucovorin was ≡ 38·3 p.c. of its wt. of folic acid for *L. casei*.

The effect of aminopterin upon the assay organisms *L. casei* and *Leuconostoc citrovorum* was examined. The inhibition index for each was determined, i.e. the ratio of the concentrations of aminopterin and metabolite at which growth, as measured by acid production, was reduced to one-half. For *Leuconostoc* this was found to be 250 and 357 when the concentrations of leucovorin were 0·30 mμg./ml. and 0·15 mμg./ml. respectively. For *L. casei* the index was about 0·30 when the organism was growing on folic acid but when leucovorin was the metabolite the index fell from 0·29 to 0·14 with rising concentration of leucovorin (Fig. 1 and Table 1).

The effect of aminopterin on the conversion to citrovorum factor of added folic acid.

Experiments were carried out to determine the effect of aminopterin upon the production of citrovorum factor during autolysis of liver tissue and upon the conversion of added folic acid (PGA) to CF. The *L. casei* assay could not be used because of the toxicity of aminopterin for this microorganism. The high inhibition index for *Leuconostoc*, however, made interference with this assay very unlikely.

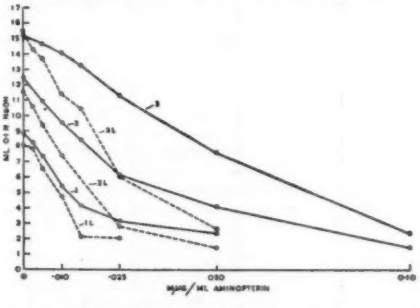

Fig. 1. The effect of "4-amino"-PGA (aminopterin) on acid production by *L. casei*.

metabolite, folic acid
curve 1 0·02 mμg./ml.
curve 2 0·04 mμg./ml.
curve 3 0·08 mμg./ml.

metabolite, Ca leucovorin
curve 1L 0·05 mμg./ml. ≡ 0·019 mμg./ml. folic acid.
curve 2L 0·10 mμg./ml. ≡ 0·038 mμg./ml. folic acid.
curve 3L 0·20 mμg./ml. ≡ 0·076 mμg./ml. folic acid.

Known amounts of folic acid were added to liver samples before autolysis and the extent of conversion to citrovorum factor was measured. The effect of aminopterin on this conversion was also determined. The results of these experiments are presented in Table 2, part 1, and summarized in Table 2, part 2. The conversion of added folic acid to CF varied in five livers from 13 to 18 p.c., the overall mean being 15 p.c. In the absence of ascorbic acid the figure was rather less. The addition of 1 mg. aminopterin reduced conversion to 0, 5 and 10 p.c. in three tests and 2 mg. reduced it to 0 to 4·6, mean 1·5 in 8 tests. The inhibitory effect of aminopterin was greater when there was no ascorbic acid in the buffer. These figures were calculated on the assumption that the aminopterin contained no folic acid. If, as seems possible, it was contaminated with folic acid the effect of aminopterin on conversion of added PGA to CF was even more marked than the tabulated figures indicate. This matter is discussed later.

TABLE 2.

Part 1.

The effect of aminopterin added before incubation on the conversion of added folic acid to leucovorin.

10 gm. liver in 50 ml. M/5 phosphate pH 7·5. No homogenization. 24 hours' incubation.

Liver No.	1 p.c. A.A.* + or −	Aminopterin added mg.	Folic acid added μg.	Leucovorin ≡ added folic acid μg.†	Leucovorin in total volume of extract μg.	Conversion of added folic acid to leucovorin $\frac{B-A}{153} \times 100$
29	+	0	0	0	24 26	
					Mean 25 (A)	
		0	58·7	153	48·5 (B) 55·0 (B)	15 20
		2 (J‡)	0	0	27 29	
					Mean 28 (A)	
		2 (J)	58·7	153	27·6 (B) 34·8 (B)	0 4·4
30	+	0	0	0	168 171	
					Mean 169·5 (A)	
		0	58·7	153	192 (B) 193 (B)	15 15
		1 (L‡)	0	0	145 128	
					Mean 136 (A)	
		1 (L)	58·7	153	151 (B)	9·8
		2 (L)	0	0	145 134	
					Mean 139 (A)	
		2 (L)	58·7	153	136 (B) 125 (B)	0 0
31	+	0	0	0	97 82	
					Mean 89·5 (A)	
		0	58·7	153	112 (B) 122 (B)	15 21

TABLE 2—*continued.*

Liver No.	1 p.c. A.A.* + or −	Aminopterin added mg.	Folic acid added μg.	Leucovorin ≡ added folic acid μg.†	Leucovorin in total volume of extract μg.	Conversion of added folic acid to leucovorin $\frac{B-A}{153} \times 100$
31	+	1 (L)	0	0	93 87	
					Mean 90 (A)	
		1 (L)	58·7	153	87 (B) 98 (B)	0 5·2
		2 (L)	0	0	94 88	
					Mean 91 (A)	
		2 (L)	58·7	153	98 (B) 94 (B)	4·6 2·0
	−	0	0	0	61 66	
					Mean 63·5 (A)	
		0	58·7	153	82·6 (B)	12·5
		1 (L)	0	0	45 46	
					Mean 45·5 (A)	
		1 (L)	58·7	153	55·1 (B) 54·1 (B)	6·3 5·6
		2 (L)	0	0	52 53	
					Mean 52·5 (A)	
		2 (L)	58·7	153	48·4 (B) 51·9 (B)	0 0
32	+	0	0	0	18·2 18·9	
					Mean 18·55 (A)	
		0	58·7	153	38·4 (B) 37·7 (B)	13·0 12·5
		2 (L)	0	0	19·7 22·7	
					Mean 21·2 (A)	
		2 (L)	58·7	153	23·0 (B) 19·5 (B)	1·2 0

TABLE 2—*continued.*

Liver No.	1 p.c. A.A.* + or −	Aminopterin added mg.	Folic acid added μg.	Leucovorin ≡ added folic acid μg.†	Leucovorin in total volume of extract μg.	Conversion of added folic acid to leucovorin $\frac{B-A}{153} \times 100$
33	+	0	0	0	98 108 101 96 103 97	
					Mean 100 (A)	
		0	58·7	153	117 124 131 118 127 122	
					Mean 123 (B)	15
		5 (L)	0	0	81 84	
					Mean 82·5 (A)	
		5 (L)	58·7	153	88·3 (B) 86·3 (B)	3·8 2·5
33	−	0	0	0	71 83	
					Mean 77 (A)	
		0	58·7	153	94·9 (B) 91·7 (B)	11·7 9·6
		5 (L)	0	0	56 54	
					Mean 55 (A)	
		5 (L)	58·7	153	48·4 (B) 46·4 (B)	0 0

After incubation measure and adjust to pH 7·0.

† 1 mg. folic acid is ≡ 100/38·3 mg. of the leucovorin used as a standard ∴ 58·7 μg. folic acid is ≡ 153 μg. leucovorin.

* Ascorbic acid.

‡J A crude preparation of "4-amino" PGA.

‡L A commercial product.

TABLE 2.

Part 2.

Summary of data presented in last column of Table 2, Part 1. Conversion of added folic acid to leucovorin: $\frac{B-A}{153} \times 100$

Liver No.	1 p.c. ascorbic acid in buffer				No ascorbic acid in buffer			
	Aminopterin mg.							
	0	1	2	5	0	1	2	5
29	15 20		0 4·4					
30	15 15	9·8	0 0					
31	15 21	0 5·2	4·6 2·0		12·5	6·3 5·6	0 0	
32	13 12·5		1·2 0					
33 (Mean of 6)	15			3·8 2·5	11·7 9·6			0 0

TABLE 3.

Part 1.

The effect of aminopterin added before incubation on the estimate of leucovorin.

10 gm. liver: 24 hours' incubation.

Liver No.	Conditions				Aminopterin added	pH after incubn.	Leucovorin† μg./gm. wet wt.
	M/5 phosphate buffer			Homogenize before incbn. + or −			
	pH	Volume ml.	1 p.c. A.A.* + or −				
26	7·5	50	+	−	0 1 mg. L‡ 5 mg. L	6·1 6·1 6·5	12·5 11·4 10·6
26	7·0	130	+	+	0 1 mg. L 5 mg. L	6·9 6·8 6·8	11·9 11·1 10·5
27	7·5	50	+	−	0 0 1 mg. L 1 mg. L	6·8 6·8 6·8 6·9	1·44 1·38 1·84 1·85

TABLE 3—*continued.*

Liver No.	Conditions				Aminopterin added	pH after incubn.	Leucovorin† µg./gm. wet wt.
	M/5 phosphate buffer			Homogenize before incbn. + or −			
	pH	Volume ml.	1 p.c. A.A.* + or −				
28	7·5	50	+	−	0	6·5	8·9
					0	6·6	8·2
					1 mg. L	6·7	6·7
					1 mg. L	6·6	7·2
28	7·0	130	+	+	0	6·9	7·2
					0	6·9	7·3
					1 mg. L	6·9	7·3
					1 mg. L	6·9	7·7
29	7·5	50	+	−	0	6·4	2·4
					0	6·4	2·6
					1 mg. J‡	6·5	2·8
					1 mg. J	6·5	2·8
					2 mg. J	6·5	2·7
					2 mg. J	6·5	2·9
30	7·5	50	+	−	0	6·0	16·8
					0	6·0	17·1
					1 mg. L	6·4	14·5
					1 mg. L	6·4	12·8
					2 mg. L	6·3	14·5
					2 mg. L	6·4	13·4
31	7·5	50	+	−	0	6·2	9·7
					0	6·3	8·2
					1 mg. L	6·4	9·3
					1 mg. L	6·4	8·7
					2 mg. L	6·4	9·4
					2 mg. L	6·5	8·8
31	7·5	50	−	−	0	6·5	6·1
					0	6·4	6·6
					1 mg. L	6·5	4·5
					1 mg. L	6·5	4·6
					2 mg. L	6·5	5·2
					2 mg. L	6·5	5·3
32	7·5	50	+	−	0	6·6	1·82
					0	6·5	1·89
					2 mg. L	6·6	1·97
					2 mg. L	6·7	2·27
	7·5	50	−	−	0	6·7	1·48
					0	6·7	1·14
					2 mg. L	6·7	1·18
					2 mg. L	6·8	1·16

TABLE 3—*continued.*

Liver No.	Conditions				Aminopterin added	pH after incubn.	Leucovorin† μg./gm. wet wt.
	M/5 phosphate buffer			Homogenize before incbn. + or −			
	pH	Volume ml.	1 p.c. A.A.* + or −				
33	7·5	50	+	−	0	6·5	9·8
						6·5	10·8
						6·5	10·1
						6·5	9·6
						6·5	10·3
						6·5	9·7
						Mean	10·0
					5 mg. L	6·8	8·1
						6·8	8·4
	7·5	50	−	−	0	6·7	7·1
						6·7	8·3
					5 mg. L	6·8	5·6
					5 mg. L	6·8	5·4

After incubation measure and adjust pH to 7·0.

* Ascorbic acid.

‡L A commercial product.

‡J A crude preparation of "4-amino" PGA.

† The Ca leucovorin which was used as a standard was ≡ 38·3 p.c. of its wt. of folic acid for *L. casei*.

TABLE 3.

Part 2.

Summary of data presented in Table 3, Part 1.

Estimate of leucovorin obtained after incubating in presence of aminopterin expressed as a percentage of the estimate made without aminopterin.

Liver No.	50 ml. buffer with 1 p.c. A.A.*			130 ml. buffer with 1 p.c. A.A.*			50 ml. buffer with no A.A.		
	Aminopterin mg.								
	1	2	5	1	2	5	1	2	5
26	91		85	93		88			
27	131								
28	81			103					
29	112	112							
30	81	82							
31	101	102					72	83	
32		114							
33			82·5						71

* Ascorbic acid.

The effect of aminopterin on the estimate of citrovorum factor, expressed as leucovorin, in the liver.

In a preliminary experiment it was found that the addition of 0·1 mg. or 0·5 mg. aminopterin to the 10 gm. samples of autolyzing liver had little or no effect upon the estimate of leucovorin. Accordingly, larger amounts, 1, 2 or 5 mg., were used. The effects of homogenizing the tissue before incubation and of omitting ascorbic acid from the buffer were also examined. One liver, No. 29, was treated with a sample of aminopterin having a lower inhibition index for *L. casei* (Table 1). It was also less toxic for *Leuconostoc* than was the aminopterin used throughout the rest of the work. The results of these experiments are presented in Table 3.

To ensure that the findings could not be attributed to any effect on the microorganism a test was made in which aminopterin was added to the liver extracts immediately prior to assay. The concentration was the same as when added before incubation. The results were as follows:

		Leucovorin, μg./gm.
Liver No. 27	no aminopterin	1·44
		1·38
	+ aminopterin before assay	1·31
		1·25
	+ aminopterin before incubation	1·84
		1·85

		Leucovorin, μg./gm.
Liver No. 28	no aminopterin	8·9
		8·2
	+ aminopterin before assay	8·9
		8·3
	+ aminopterin before incubation	6·7
		7·2

It is obvious that the increased measure of leucovorin with liver No. 27 and the decreased measure with liver No. 28 arising out of incubation with aminopterin could not be ascribed to any effect in the assay. The same is true with the other liver extracts.

For three of the livers examined the presence of aminopterin caused a rise in the measurement of leucovorin. These livers were relatively deficient in leucovorin and, therefore, had a higher aminopterin to leucovorin ratio than was the case with the normal livers. A possible explanation of the findings is that the aminopterin contained some impurity, initially inactive in the *Leuconostoc* assay, but developing activity on incubation. It has been reported that commercial

preparations of aminopterin may contain as much as 20 p.c. of folic acid (Nichol, Zakrzewski and Welch, 1953). However, the manufacturers of the aminopterin used by us have stated that it is not less than 97·0 p.c. pure.

The data in Table 2 show that in the presence of aminopterin a small percentage of added folic acid was at times converted to citrovorum factor. If we postulate that the aminopterin contained 3 p.c. folic acid the figures in Table 3, part 2, must be reduced, and also those figures in Table 2 which express the percentage conversion of added folic acid to citrovorum factor, in the presence of aminopterin.

An example of the required calculation follows:

The results which have been given for liver No. 33 can be recalculated on the assumption that the aminopterin contained 3 p.c. folic acid. It is first necessary to recalculate the percentage conversion to leucovorin of added folic acid (Table 2, part 1). The 58·7 μg. folic acid which was added to the 10 gm. liver samples would have been equivalent to $58{\cdot}7 \times 100/38{\cdot}3 = 153$ μg. of Ca leucovorin if it had been completely converted to citrovorum factor. The actual conversion was

$$\frac{B-A}{153} \times 100 = 3{\cdot}8 \text{ p.c. and } 2{\cdot}5 \text{ p.c.}$$

in two tests.

If the 5 mg. aminopterin contained 150 μg. folic acid (≡ 392 μg. leucovorin) the conversion to leucovorin was

$$\frac{B-A}{153+392} \times 100 = 1{\cdot}06 \text{ p.c. and } 0{\cdot}70 \text{ p.c. in two tests. Mean } 0{\cdot}88 \text{ p.c.}$$

Turning to Table 3 we find:

(1) leucovorin in 10 gm. liver = 100 μg.

(2) leucovorin in 10 gm. liver after adding 5 mg. aminopterin ... ... = 82·5 μg.

From (2) must be subtracted the leucovorin derived from the aminopterin, i.e.

0·88 p.c. of 392 = 3·5 μg.

leaving 79μg.

The figure 82·5 in Table 3, part 2, would therefore become 79.

It can be calculated that if the aminopterin contained 5 p.c. folic acid this figure would be 78·5 and even a 20 p.c. contamination with folic acid would reduce it only to 78.

To reduce the value 131 p.c. for liver No. 27 to 100 p.c. would require a 5·6 p.c. conversion to leucovorin of the 3 p.c. folic acid in 1 mg. aminopterin.

We are probably justified in concluding that with ascorbic acid in the buffer the total citrovorum factor in the liver was reduced by not more than one-quarter as a result of autolyzing in the presence of aminopterin. The reduction was somewhat greater without ascorbic acid. The remainder of the CF in the liver

was apparently derived from precursors by a process which was not affected by aminopterin. The liberation of citrovorum factor from its polyglutamates might be one such process.

SUMMARY.

The inhibitory effect of aminopterin on the growth of *Lactobacillus casei* and *Leuconostoc citrovorum* was examined and inhibition indices were determined.

The concentration of citrovorum factor in liver, measured after 24 hours' autolysis, was reduced by not more than one-quarter in the presence of aminopterin.

Aminopterin markedly inhibited the conversion of added folic acid to citrovorum factor by autolyzing liver.

REFERENCES.

Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H. (1950): J. biol. Chem., 185, p. 399.
Dawbarn, M. C., Hine, D. C., and Smith, J. (1958): Austral. J. exp. Biol., 36, p. 511.
Dinning, J. S., Sime, J. T., Work, P. S., Allen, B., and Day, P. L. (1957): Arch. Biochem. and Biophys., 66, p. 114.
Futterman, S. (1957): J. biol. Chem., 228, p. 1031.
Futterman, S., and Silverman, M. (1957): *Ibid.*, 224, p. 31.
Hendlin, D., Koditschek, L. K., and Soars, M. H. (1953): J. Bact., 65, p. 466.
Jukes, T. H. (1953): Fed. Proc., 12, p. 633.
Nichol, C. A., Anton, A. H., and Zakrzewski, S. F. (1955): Science, 121, p. 275.
Nichol, C. A., and Welch, A. D. (1950): Proc. Soc. exp. Biol. and Med., 74, pp. 52 and 403.
Nichol, C. A., Zakrzewski, S. F., and Welch, A. D. (1953): *Ibid.*, 83, p. 272.
Osborn, M. J., Freeman, M., and Huennekens, F. M. (1958): *Ibid.*, 97, p. 429.
Reid, B. L., and Couch, J. R. (1955): Arch. Biochem. and Biophys., 56, p. 388.
Sauberlich, H. E. (1949): Fed. Proc., 8, p. 247.
Sauberlich, H. E. (1949): Arch. Biochem., 24, p. 224.
Seeger, D. R., Cosulich, D. B., Smith, J. M., and Hultquist, M. E. (1949): J. Amer. chem. Soc., 71, p. 1753.
Seeger, D. R., Smith, J. M., and Hultquist, M. E. (1947): *Ibid.*, 69, p. 2567.

FOLIC ACID ACTIVITY IN THE LIVER OF SHEEP

3. THE EFFECT OF VITAMIN B_{12} DEFICIENCY ON THE CONCENTRATION OF FOLIC ACID AND CITROVORUM FACTOR

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Since the discovery that a haematological remission can be secured in pernicious anaemia by the administration of either folic acid or vitamin B_{12} the interrelationship of these two vitamins has been studied extensively. No attempt will be made here to consider the voluminous literature on the subject, but several reviews should be mentioned (Darby, 1947; Welch and Heinle, 1951; Jukes, 1953; Shive, 1953; Witts, 1956).

We wish to report findings made in this laboratory concerning the effect of vitamin B_{12} deficiency on the concentration of folic acid and citrovorum factor in the liver of sheep.

METHODS.

The concentration of vitamin B_{12} in the liver was determined by means of a microbiological assay with *Lactobacillus leichmannii* (Dawbarn and Hine, 1954; Hine and Dawbarn, 1954).

Folic acid activity and citrovorum factor were measured during an investigation described in the first paper in this series (Dawbarn, Hine and Smith, 1958). These are the same livers and have been given the same numbers as in that paper. The concentrations of total folic acid and of citrovorum factor have been expressed in terms of folic acid (pteroylglutamic acid) and Ca leucovorin which were used as standards in assays with *Lactobacillus casei* and *Leuconostoc citrovorum*. The activity of the Ca leucovorin for *L. casei* was estimated to be equivalent to 38·3 p.c. of its weight of folic acid. The results of the assays are presented in Table 1. They comprise estimates which were made on extracts of liver after autolysis in the presence of ascorbic acid.

Thirty livers were examined. Fourteen of these were obtained from retail stores and the remainder from laboratory animals of known history which is described in footnotes to Table 1.

RESULTS AND DISCUSSION.

The livers have been divided into three groups: group I, vitamin B_{12} from 0·19 to 1·87 μg./gm.; group II, vitamin B_{12} from 0·032 to 0·136 μg./gm.; group III will be discussed later.

The concentration of folic acid in the first two groups was from 3·1 to 9·9, mean 6·4 μg./gm. and from 0·62 to 1·83, mean 1·18 μg./gm.

The citrovorum factor varied from 5·6 to 21, mean 13·3 μg. leucovorin/gm. in group I, and from 0·69 to 3·2, mean 1·89 μg. leucovorin/gm. in group II.

The mean folic acid concentration in group II was less than one-fifth the mean in group I; for leucovorin the ratio was one to seven.

The correlation coefficients between vitamin B_{12} and folic acid and between vitamin B_{12} and leucovorin were calculated for the two groups separately. They were not significant.

The concentrations of folic acid and citrovorum factor in the livers in group I are considered to be normal. The group included one liver which was obtained from a sheep subsisting on a cobalt-deficient ration. The vitamin B_{12} content was 0·19 μg./gm.

TABLE 1.

Vitamin B_{12}, folic acid and leucovorin in liver.

Liver No.	Sheep No.	μg./gm. wet wt.			
		B_{12}[1]	Folic acid	Leucovorin[2]	
		Group I			
11		1·87	5·5	12·9	A
25		1·87	6·9	16·5	A
31		1·84	4·4	8·9	A
16		1·80	5·4	7·4	B
18		1·70	9·9	13·2	A
34		1·56	9·2	19·5	A
14		1·51	4·7	10·1	B
7		1·47	9·3	21	A
26		1·45	6·9	12·5	A
17		1·41	7·9	18·0	A
9		1·38	3·1	5·6	B
30		1·29	7·0	16·9	A
19		1·21	5·8	11·3	A
5		1·17	6·5	13·1	A
20		1·10	6·9	14·9	A
33		0·98	5·2	10·0	A
23		0·49	5·2	13·2	A
24	534*	0·188	4·8	13·7	C
Mean			6·4	13·3	

Group II

22	272†	0·136	1·00	1·53	
29	265*	0·070	1·83	2·5	C
13	460	0·066	1·73	3·2	D
6	211*	0·065	0·97	1·69	C
15	040*	0·060	1·33	2·5	C
32	545*	0·050	1·15	1·85	C
35	438*	0·050	1·43	2·5	C
12	140	0·038	0·78	1·05	D
8	096*	0·033	0·62	0·69	C
27	039*	0·032	0·97	1·41	C
Mean			1·18	1·89	

Group III

Sheep on restricted food intake Vitamin B_{12} by injection.

28	329 pair fed with 039	1·97	4·5	8·5	C
36	363 pair fed with 438	0·94	4·5	7·0	C

A Liver from retail store.
B Liver from sheep on wheaten hay chaff, 10 mg. cobalt per week *per os*.
C Liver from sheep on cobalt deficient diet of wheaten hay chaff and gluten.
* Food intake reduced due to failure of appetite.
D Liver from sheep on cobalt deficient pasture at Robe, South Australia, then on cobalt deficient wheaten hay chaff No. 140 for 4 days; No. 460 for 19 days.
† Sheep No. 272: as for group C but during the week before slaughter received 1 mg. cobalt *per os* daily.
Correlation:
Between vitamin B_{12} and folic acid
Group I $r = 0·25$, not significant.
Group II $r = 0·21$, not significant.
Between vitamin B_{12} and leucovorin
Group I $r = 0·04$, not significant.
Group II $r = 0·21$, not significant.
[1] Pure crystalline vitamin B_{12} (Glaxo) was used as a standard.
[2] The Ca leucovorin which was used as a standard was $\equiv 38·3$ p.c. of its wt. of folic acid for *L. casei*.

The livers in the second group were all from sheep which were suffering from vitamin B_{12} deficiency induced by a shortage of cobalt in the diet. One sheep which had received cobalt by mouth for a week before slaughter had a liver vitamin B_{12} content of 0·136 μg./gm. The concentration in the rest of the livers was below 0·1 μg./gm.

The daily food intake of 7 out of the 10 sheep in group II was measured throughout the period in which they were subsisting on the deficient ration. In every case food intake had fallen due to the failure of appetite, which is one of the most obvious signs of cobalt deficiency (Marston, 1952). It was thought that the decline in folic acid and citrovorum factor in the liver might be due to this cause rather than to vitamin B_{12} deficiency *per se*.

To clarify this point livers were obtained from sheep whose food intake

was restricted but which were not vitamin B_{12} deficient. Two pairs of sheep were fed the same cobalt-deficient ration. One of each pair was vitamin B_{12} deficient and was fed *ad libitum*. The food intake of the other was equalized with that of the deficient animal, but it was given vitamin B_{12} by injection. The results for the two sheep with restricted food intake but adequate vitamin B_{12} are presented in Table 1 (group III). Their folic acid and leucovorin contents were within the normal range.

It would seem that at some point in the development of vitamin B_{12} deficiency when the concentration in the liver was below 0·19 μg./gm. the concentrations of folic acid and citrovorum factor fell precipitately. There was no suggestion of a gradual fall with failing vitamin B_{12}.

The proportion of the total folic acid activity which could be attributed to citrovorum factor was 80 p.c. in group I and 61 p.c. in group II, calculated from the means (Table 1). The corresponding figures for the paired animals were 56 and 67 p.c. for the vitamin B_{12} deficient sheep and 72 and 60 p.c. for the other two. The higher proportion of citrovorum factor in the livers in group I is, therefore, probably not due to their higher vitamin B_{12} content.

The recovery of folic acid added before incubation and its conversion to citrovorum factor were examined in five livers, Nos. 30, 31 and 33 from group I and Nos. 29 and 32 from group II. The detailed results have been presented in the preceding communications. They may be briefly summarized here.

Recovery of folic acid added before incubation.

$$\frac{\text{Total F.A. found} \times 100}{\text{Total F.A. expected}}$$

No.	Normal livers	No.	B_{12} deficient livers
30	89	29	90
	85		73
31	88		81
	90		75
33	92	32	86
	84		86
			89

Conversion of added folic acid to citrovorum factor.
Per cent.

No.	Normal livers	No.	B_{12} deficient livers
30	15	29	15
	15		20
31	15	32	13
	21		12·5
33 (mean of 6 determinations)	15		

There is no marked difference between the normal and deficient livers in either of these tests.

SUMMARY.

The vitamin B_{12}, folic acid and citrovorum factor concentrations in the livers of thirty sheep were determined.

In group I the vitamin B_{12} concentration varied from 0·19 to 1·87 μg./gm. of liver, the folic acid from 3·1 to 9·9 and the citrovorum factor, expressed in terms of the Ca leucovorin used as a standard, from 5·6 to 21 μg./gm.

In group II the vitamin B_{12} concentration varied from 0·032 to 0·136 μg./gm. of liver, the folic acid from 0·62 to 1·83 and the citrovorum factor, as leucovorin, from 0·69 to 3·2 μg./gm.

There was no significant correlation between vitamin B_{12} and folic acid or between vitamin B_{12} and citrovorum factor in either group.

It is suggested that the concentration of folic acid and citrovorum factor in the liver fell precipitately when the vitamin B_{12} concentration dropped below 0·19 μg./gm.

This fall was not due to the restricted food intake which occurred as a result of the vitamin B_{12} deficiency.

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REFERENCES.

Darby, W. J. (1947): Vitamins and Hormones, 5, p. 119.
Dawbarn, M. C., and Hine, D. C. (1954): Austral. J. exp. Biol., 32, p. 1.
Dawbarn, M. C., Hine, D. C., and Smith, J. (1958): *Ibid.*, 36, pp. 511 and 529.
Hine, D. C., and Dawbarn, M. C. (1954): *Ibid.*, 32, p. 641.
Jukes, T. H. (1953): Fed. Proc., 12, p. 633.
Marston, H. R. (1952): Physiol. Rev., 32, p. 66.
Shive, W. (1953): Fed. Proc., 12, p. 639.
Welch, A. D., and Heinle, R. W. (1951): Pharmacol. Rev., 3, p. 345.
Witts, L. J. (1956): Brit. med. Bull., 12, p. 14.

ADVERSE EFFECT OF FILTRATION ON THE IMMUNIZING POWER OF STAPHYLOCOCCAL TOXIN AND TOXOID

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Fisher (1957) has recently shown that formalin toxoidation greatly impairs the antigenicity of staphylococcal alpha toxin.

This paper presents evidence which shows that the antigenicity of toxin is also adversely affected by filtration. Experiments are also recorded, the results of which suggest that filtration affects the protective antigenicity of toxin more than its haemolytic and lethal actions. By analogy, it might be assumed that the antigenicity of toxoid is also impaired by filtration, and the results of other tests described below support this assumption.

Materials and Methods.

Experimental animals.—White mice weighing 18-20 gms. were used.

Staphylococcal toxic supernatants.

Cultures of strain "CN56" or of strain "Mills" were grown for 3 days on modified Dolman's (1933) medium in an atmosphere of oxygen containing 20 p.c. CO_2. They were then centrifuged for 1 hour at 2,000 r.p.m. and the supernatants drawn off. 0·02 p.c. thiomersal was added as a preservative. These supernatants are referred to as unfiltered toxins.

Staphylococcal toxic filtrates.

Part of both toxic supernatants was Seitz filtered using 3·6 cm. Hormann Ekwip filter pads, Grade D-9. The filtrates are referred to as Seitz filtered toxins. Part of the toxic supernatant CN56 was filtered through a membrane filter with an average pore diameter of 200 $m\mu$ and this is referred to as membrane filtrated toxin.

A further portion of the toxic supernatant CN56 was filtered through a "Corning" fritted glass filter (fine) pore size 4-5·5 μ. This is referred to as glass filtered toxin.

Staphylococcal toxoid.

This was prepared as follows: staphylococcal toxic supernatant (unfiltered toxin) was mixed with 0·35 p.c. formalin and incubated at 37° C. until toxicity had been destroyed as shown by complete loss of haemolytic power for rabbit and sheep cells when tested at 1:10 dilution. This took up to 30 days. Part of this unfiltered toxoid was Seitz filtered and part glass filtered in the same manner as described for toxins.

Immunization of mice.

Two subcutaneous injections were given one week apart. A dose of toxin (both unfiltered and filtered) was 0·5 ml. of 1 : 50 dilution unless otherwise stated. A dose of toxoid

(both unfiltered and filtered) was 1 ml. of undiluted material. The mice were challenged by the intraperitoneal route 14 days after the second immunizing injection.

Preparation of challenge suspension of staphylococci.

A stable living bacterial pool was prepared by a modification of the method described by Yurchenco, Piepoli and Yurchenco (1954) for the preparation of pools of living cells of other bacterial species.

A recently isolated coagulase-producing strain, S1531, was grown overnight on nutrient agar. A heavy suspension in 5 p.c. hog gastric mucin was prepared and 5 ml. volumes were dispensed into ½-oz. screw cap (McCartney) bottles and immediately frozen in alcohol and dry ice. These were then stored in the dry ice box until required.

Preliminary tests on samples from this pool after being thawed out in a water bath at 37° C. showed that the intraperitoneal injection of 1 ml. of 1 : 60 dilution in Boake's (1956) "clotting mixture" killed approximately 80-90 p.c. of unimmunized mice. "Clotting mixture" consisted of 1 p.c. human fibrinogen and 1 p.c. fresh human plasma in normal saline. This challenge dose contained approximately $1{\cdot}5 \times 10^8$ living staphylococci (as determined by the method of Miles and Misra, 1938). No falling off in viable content or in virulence was noted over a period of many months..

The challenge dose in "clotting mixture" was prepared fresh immediately before use.

Alpha haemolysin, combination of toxin with antitoxin.—Tests were carried out by the methods of Burnet (1931).

Mouse sera.

Samples of blood were taken from the tails of approximately 50 p.c. of mice of each group and pooled. After separation from the clot, the mouse sera were tested without preliminary heat inactivation for antihaemolysin (Burnet, 1931) against the international standard staphylococcal alpha antihaemolysin.

Toxicity tests.—These were carried out in mice by injecting graduated doses of either unfiltered or of filtered toxin intraperitoneally.

RESULTS.

Tests with unfiltered and filtered toxin.

Mice immunized with unfiltered toxin always appeared to be better protected than those receiving the same dose of Seitz filtered material.

The results of a representative experiment are shown in Table 1. In this test in which Toxin "Mills" was used there is a statistically significant differ-

TABLE 1.

Comparison of protection against lethal challenge I.P. with living Staphylococci of mice immunized with Seitz filtered and unfiltered toxin (Strain "Mills").

Immunizing agent	Day of death after challenge of individual mice	Proportion of survivors 7 days after challenge.
Unfiltered toxin	1, 2	11/13
Seitz filtered toxin	1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 2	4/15
Nil (Controls)	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 3	3/14

Immunized mice given two subcutaneous injections each of 0·5 ml. of 1 : 50 toxin with an interval of 7 days between injections and challenged 14 days after the last injection together with the non-immunized controls.

ence between the numbers of survivors in the two immunized groups. As shown in the Table, nearly all deaths both in immunized and in unimmunized animals occurred within 48 hours of challenge. Early death appears to be the rule in mice after intraperitoneal challenge with living staphylococci whose virulence has been enhanced by suspension in "clotting mixture". For this reason the time of death of individual animals will not be included in the description of other similar experiments.

A series of tests was carried out in which the immunizing potency of unfiltered toxin was compared directly with that of membrane filtered, glass filtered and Seitz filtered portions of the same toxin. The results of a representative experiment in which Toxin CN56 was used are shown in Table 2. In this test

TABLE 2.

Comparison of immunizing power of filtered and unfiltered toxins (CN56) in mice against subsequent challenge I.P. with living Staphylococci.

Immunizing agent	Alpha antihaemolysin titre of mouse sera 1 day before challenge	Proportion of mice surviving 7 days after challenge
Unfiltered toxin	3 units	14/17
Membrane filtered toxin	3 units	13/20
Glass filtered toxin	1·5 units	9/20
Seitz filtered toxin	<0·25 units	4/20
Nil (Controls)	<0·25 units	2/22

Immunized mice given 2 subcutaneous injections each of 0·5 ml. of 1 : 50 toxin with an interval of 7 days between injections and challenged 14 days after the last injection together with the non-immunized controls. The pooled serum from approximately 50 p.c. of mice in a group was used for estimating the antitoxin titre.

each group of immunized mice, except that to which Seitz filtered toxin was administered, was protected to a statistically significant degree as compared with the unimmunized animals. Further, there is a statistically significant difference between the degree of protection of the unfiltered and the glass filtered toxin groups.

Another finding of interest in this experiment was the correlation shown between the antihaemolysin titres of the mouse sera of the different groups before challenge, and the degree of protection as indicated by the proportion of survivors. As reference to Table 3 will show, such correlation may be (and often is) absent.

All the tests carried out showed the same general pattern amongst the different immunization groups. Protection was strongest in the group receiving unfiltered toxin and weakest in the group immunized with Seitz filtered toxin.

The degree of protection shown by the mice receiving membrane filtered and glass filtered toxin was intermediate between that of the unfiltered and Seitz filtered groups. There appeared to be little difference in the immunizing

TABLE 3.

Comparison of the immunizing power of Seitz filtered and unfiltered toxin CN56 using graded doses of toxin.

Immunizing agent	Dilution used	Antitoxin titre of mouse sera 1 day before challenge	Proportion of mice surviving 7 days after challenge
Unfiltered toxin	1 : 70	0·75 units	11/16
Unfiltered toxin	1 : 90	0·75 units	11/16
Seitz filtered toxin	1 : 50	1·5 units	8/15
Seitz filtered toxin	1 : 70	0·25 units	3/11
Seitz filtered toxin	1 : 90	0·3 units	2/16
Nil (Controls)	—	—	2/15

Immunized mice given 2 subcutaneous injections each of 0·5 ml. of toxin dilution with an interval of 7 days between injections and challenged 14 days after last injection together with the non-immunized controls.

power of the membrane filtered and the glass filtered toxins, although the combined results in four consecutive tests (the details of which will not be described) slightly favoured the former – 46 survivors out of 72 mice as against 32 out of 68. There was a total of 12 survivors among 73 unimmunized mice used as controls.

In an endeavour to estimate the loss of protective antigenicity during Seitz filtration, graded doses of unfiltered and Seitz filtered toxin were administered to different groups of mice. The immunization and subsequent challenge were carried out according to the routine described. The protocol of a representative experiment is shown in Table 3. The results which were consistent with those of other tests suggest that at least one-half of the protective antigen was lost during filtration.

The results of an experiment in which the lethal potency of unfiltered and Seitz filtered toxins (Strain CN56) was compared are shown in Table 4. The filtered toxin was approximately 20 p.c. less potent than the unfiltered.

TABLE 4.

Comparison of toxicity (lethal power) of Seitz filtered and unfiltered toxin, CN56.

Dilution of toxin	Proportion of mice surviving 5 days after injection of toxin	
	Unfiltered	Seitz filtered
1 : 10	—	0/10
1 : 14	1/10	4/10
1 : 18	4/10	5/10
1 : 25	7/10	9/10
1 : 36	10/10	—

Mice were injected intraperitoneally with 0·5 ml. amounts of toxin dilution in saline shown in the first column. The test was terminated after 3 days.

Another test using unfiltered and Seitz filtered toxin "Mills" gave concordant results.

Titrations of alpha haemolysis and estimations of combining power with staphylococcal antitoxin were carried out a number of times in parallel on unfiltered and Seitz filtered toxins. The results of tests carried out on the one day for alpha haemolysis are shown in Table 5 and for combining power with standard staphylococcal antitoxin in Table 6. The filtered "Mills" toxin contained

TABLE 5.

Comparison of alpha haemolysin titres of unfiltered and Seitz filtered toxins (50% haemolysis).

Strain	Unfiltered toxin	Seitz filtered toxin
CN56	1 : 854	1 : 640
"Mills"	1 : 640	1 : 427

After addition of rabbits R.B. Cells (final concentration 1 p.c.) tubes were shaken well and placed in water bath at 37°C. Tubes shaken after 30 minutes, then removed after further 30 minutes, centrifuged and read.

TABLE 6.

Comparison of combining power of unfiltered and Seitz filtered toxins with standard staphylococcal antitoxin.

Strain	Treatment of toxin prior to test	No. of volumes of antitoxin (1·6 units/ml.) required to prevent haemolysis by 1 volume of toxin
CN56	Unfiltered	13
CN56	Seitz filtered	11
"Mills"	Unfiltered	8
"Mills"	Seitz filtered	6

Mixtures of toxin and antitoxin stood in water bath at 37°C. for 30 minutes. R.B.Cs. (final concentration 1 p.c.) added, tubes shaken and returned to water bath for 30 minutes. Tubes then shaken again and removed after further 30 minutes, centrifuged and read. 50 p.c. haemolysis taken as end point.

two-thirds of the haemolysin and three-quarters of the combining power of the unfiltered. The corresponding proportions for the CN56 toxin were three-quarters and four-fifths.

Part of an unfiltered toxin (CN56) was centrifuged at 4° C. for 30 minutes at 16,000 r.p.m. The supernatant and the resuspended deposit were then compared with the untreated portion for protective potency by the methods described above. The results of this experiment are shown in Table 7.

The absence of an appreciable loss of antigenicity after high speed centrifugation is confirmed by the fact that so little protective antigen was present in the deposit. Further, no dermonecrosis whatever was produced in the mice by injecting the amount of deposit shown in Table 7.

TABLE 7.

Effect of high speed centrifugation on the antigenicity of unfiltered toxin.

Immunizing agent	Dilution used	Antitoxin titre of mouse sera 1 day before challenge	Proportion of mice surviving 7 days after challenge
Unfiltered toxin	1 : 50	0·5 units	16/20
Supernatant of centrifuged unfiltered toxin	1 : 50	0·5 units	18/20
Resuspended deposit	1 : 2	0·125 units	8/20
Nil (Controls)	—	<0·125 units	3/20

Immunized mice given two subcutaneous injections each of 0·5 ml. of toxin diluted 1 : 50 or of resuspended deposit diluted 1 : 2 after resuspending to original volume. Interval of 7 days between injections, mice challenged 14 days later.

Active immunization tests with unfiltered and filtered staphylococcal toxoid.

Owing to the marked loss in antigenicity of the protective factor during toxoiding (Fisher, 1957) it is necessary to give comparatively large doses of toxoid to show protection in immunized groups. The results of an experiment in which a statistically highly significant degree of protection was obtained in the group immunized with unfiltered toxoid are shown in Table 8. Whilst there appears to be some protection in the group immunized with glass filtered toxoid, there is little or none in the Seitz filtered toxoid group. This test is representative of others in which mice were immunized with unfiltered and filtered toxoids. The order of immunity in the different groups was the same as that in the tests in which non-filtered and filtered toxins were compared, although the protection obtained even with unfiltered toxoids was usually considerably less.

Part of an unfiltered toxoid was centrifuged at 4° C. for 35 minutes at 4,800 r.p.m. The supernatant was then compared with the untreated portion for protective potency by the routine described above. In the three tests carried out no statistically significant difference was found in the immunizing power of the two portions of toxoid.

DISCUSSION.

Marked reduction in the immunizing power of Seitz filtered toxin as compared with that of unfiltered toxin was evident in every test carried out. Filtration through sintered glass or membranes regularly reduced the immunizing potency of toxin but to a lesser degree. On the other hand, high speed centrifugation had no effect on its antigenicity.

Fisher (1957) has already suggested, on the basis of variable alpha antihaemolysin titres in mice immunized with toxin, that the haemolysin and protective factor are separate antigens.

Results such as those shown in Tables 2 and 3 when compared with each other and considered together with those of Tables 4, 5 and 6 support Fisher's view and suggest further that the protective factor in staphylococcal toxin is also separate from the lethal factor.

The fact (exemplified in Table 8) that mice injected with Seitz filtered toxoid and showing little or no protection may have an anti-haemolysin titre at least as high as those dosed effectively with unfiltered toxoid lends further support to this suggestion.

TABLE 8.

Comparison of immunizing power of filtered and unfiltered toxoids (Batch 79) in mice against challenge I.P. with living staphylococci.

Immunizing agent	Antitoxic titre of mouse sera 1 day before challenge	Proportion of mice surviving 7 days after challenge
Toxin CN56	0·75 units	15/18
Toxoid unfiltered	0·25 units	12/17
Toxoid glass filtered	<0·125 units	6/19
Toxoid Seitz filtered	0·3 units	4/17
Nil (Controls)	<0·125 units	2/20

Mice immunized with toxin were given 2 injections each of 0·5 ml. (1 : 50 dilution). Toxoid mice were given two doses each of 1 ml. undiluted toxoid. Both toxin and toxoid were given subcutaneously with an interval of 1 week between injections. All mice were challenged I.P. 12 days after the second immunizing injection.

As filtration, especially through asbestos pads, so markedly affects the protective antigenicity of toxin, it was only to be expected that it would have a similar effect on toxoid. With a course of two immunizing injections, the protection shown in groups receiving even unfiltered toxoid is not always of a high order, but it is clear that the pattern of protection is just the same as with unfiltered and filtered toxin.

The work reported above has practical implications both as regards active immunization of humans and the preparation of staphylococcal antitoxin.

In the preparation of toxoid for human use, staphylococcal toxic supernatant is first treated with formalin and then filtered – often through asbestos pads. Each of these procedures adversely affects the protective antigen. As unfiltered toxoid already contains sufficient formalin to kill bacteria, it may be that means will be found eventually to make staphylococcal toxoid acceptable for humans without the use of bacterial filters. In the meanwhile, glass or membrane filtration might well take the place of Seitz filtration.

After a preliminary course with an improved toxoid, the addition of a small amount of toxin to the toxoid might even be practicable.

As filtered toxoid is relatively such a poor immunizing agent as regards the production of protective antibody, immunization of horses should be started with unfiltered toxoid and continued with unfiltered toxin.

Although there is an international reference standard antitoxin available, staphylococcal antitoxin is standardized simply by an *in vitro* test – its antihaemolysin titre. It follows from Fisher's (1957) observations and from the work now reported that one serum may actually contain more protective antibody than another, having the same or even a greater amount of haemolysin than the first. Therefore, unfiltered toxoids or toxins should always be preferred to filtrates, even though the latter may stimulate the production of antitoxins of high titres judged by their antihaemolysin unitage.

SUMMARY.

Filtration of staphylococcal toxic supernatants (unfiltered toxins) and of staphylococcal toxoids reduces the content of protective antigen.

Seitz filtration is definitely more harmful than filtration through sintered glass or through membrane filters.

Filtration of toxin probably results in a proportionately greater loss of its protective factor than of its lethal factor and alpha-haemolysin.

The implications of these findings as regards the active immunization of humans and the production of more effective anti-sera in horses is discussed.

REFERENCES.

Boake, W. C. (1956): J. Immunol., 76, p. 89.
Burnet, F. M. (1931): J. Path. Bact., 34, p. 471.
Dolman, C. E. (1933): J. Amer. med. Assoc., 100, p. 1007.
Fisher, S (1957): Nature, 180, p. 1479.
Miles, A. A., and Misra, S. S. (1938): J. Hyg., 38, p. 732.
Yurchenco, J. A., Piepoli, C. R., and Yurchenco, M. C. (1954): Applied Microbiol., 2, p. 53.

THE QUANTITATIVE ESTIMATION OF PLASMA CORTICOSTEROIDS BY A MODIFIED CHROMATOGRAPHIC TECHNIQUE

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The development of quantitative chromatographic methods for the determination of individual adrenal corticosteroids in biological fluids has opened the way for precise investigation of adrenal function in normal and diseased states. In the selection of a suitable method one has to consider the quantity of sample available, the expected number, nature and quantity of the individual steroids, and the extractable impurity. The final choice then depends on the reproducibility, specificity and accuracy of the method.

In a study of the corticosteroid secretion of the adrenal gland in sheep in different experimental circumstances, it was found necessary to develop a standardized procedure for the chromatographic analysis of corticosteroids in biological fluids. Several modifications of the original Bush methods (Bush, 1952, 1952-53; Bush and Sandberg, 1953) permit satisfactory estimation of cortisol, cortisone, corticosterone and aldosterone in relatively small amounts of plasma or other body fluids with a recovery above 90 p.c. The procedure has been successfully applied to sheep adrenal, venous and peripheral arterial plasma, human peripheral arterial plasma, and extracts of sheep urine and parotid saliva. In this communication only the plasma analyses are described in detail. The extraction from urine and saliva was done as described by Ayres, Garrod, Simpson and Tait (1957) up to the commencement of chromatography. The separation was then effected as for plasma. Although many basic steps have been described by others (Bush, 1952, 1954; Zaffaroni, 1953; Eberlein and Bongiovanni, 1955; Garrod, Simpson and Tait, 1956; Neher and Wettstein, 1956; Borth, 1956; Ayres, Garrod, Simpson and Tait, 1957) it is felt that a detailed description of the method used in this laboratory will be of considerable value to those wishing to study adrenocortical function by the use of direct corticosteroid assay.

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Materials and Apparatus.

All solvents are purified and distilled not longer than 24 hrs. before use.

Ethyl acetate (Commonwealth Sugar Refineries, Aust.). Commercial grade, distilled and the fraction at 76° C. collected. The distillate was washed once with an equal volume of 5 p.c. sodium carbonate, dried over calcium chloride and then refluxed for 2 hrs. and distilled at 76° C.

Ether (Woolwich-Elliott anaesthetic). Kept on $CaCl_2$. Refluxed for 2 hrs. distilled at 34° C. The distillate washed once with ¼ volume of half-saturated ferrous sulphate, followed by one washing with an equal volume of water. Then washed once with ¼ vol. of half-saturated sodium carbonate and three washings with equal amounts of water. It was dried overnight over $CaCl_2$ and washed with water until neutral (pH 6-7).

Methanol. Analytical reagent. Refluxed 1 l. with 2·5 gm. of magnesium powder for 2 hrs. and distilled.

Ethanol. Analytical reagent. Refluxed 1 l. with 2·5 gm. of magnesium powder for 2 hrs. and distilled.

Toluene (British Drug Houses). Laboratory reagent (sulphur free). 1 l. washed with 0·1 volume of concentrated H_2SO_4 until acid colourless, temperature kept under 30° C. Washed twice with 0·2 volumes of water and once with 0·2 volumes of 10 p.c. sodium carbonate followed with another water wash. Dried over $CaCl_2$, filtered and distilled at 110·5° C.

Benzene (British Drug Houses). Laboratory reagent. Treated in the same way as toluene except that 0·15 vol. of concentrated H_2SO_4 used.

Petroleum ether. Analytical reagent B.P. (80-100) used untreated.

Pyridine. Analytical reagent. Kept on KOH pellets overnight, refluxed for 1 hr. Distilled at 115·5° C.

Acetic anhydride. Analytical reagent. Refluxed for 1 hr. Distilled between 135-140° C.

Sudan III (Ciba Ltd., Basle, Switzerland). Saturated solution in hexane.

Silica gel (Davison Chemical Co., Maryland, U.S.A.). Mesh 60-200 grade 950, used untreated.

Nitrogen (Commonwealth Industrial Gases, Sydney, N.S.W.). Oxygen free.

Paper (Reeve Angel & Co., London). Whatman No. 2 chromatography grade, 2·5 cm. strips and sheets cut into 5 cm. strips. The 5 cm. strips were washed by descending chromatography with a mixture of ethylacetate-methanol (2:1) for 48 hrs. All the strips before use were divided into 4 sections lined 2·5 cm. apart. The steroid was applied on the furthest section lined across the strip. The sections were folded so that the solvent ran first horizontally as seen in the diagram (Fig. 1).

Pump (Rud Browne Co., Melbourne). This type of vacuum pump does not require a vapour trap when used with volatile solvents.

Steroids (Ciba Ltd., Basle, Switzerland). Chromatographed to check purity. Standards made up in distilled ethyl alcohol and stored at — 15° C. in amber, narrow-necked flasks with a double stopper.

Radioactive steroids (U.S. Public Health Service, Endocrinology Section). Cortisol -4-C^{14} and corticosterone -4-C^{14} checked for chromatographic purity. Standards prepared in ethyl alcohol.

Ultraviolet light sources. For examination of sodium hydroxide treated strips, a medium pressure mercury arc was used (Hanovia Model II B). For detection of natural steroid fluorescence the Hanovia "Chromatolite" (maximum emission 2537 A.U.) was used.

Measurement of radioactivity. Phillips model preset counting scaler with two types of mica end-window G.M. tube — one with effective diameter 1·8 cm. for planchette counting, and the other with effective diameter 2·8 cm. for measurement of radioactivity on the paper strips.

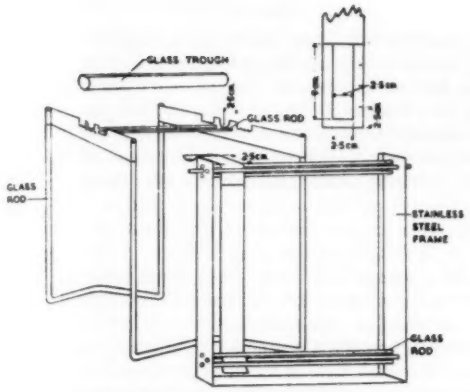

Fig. 1. Chromatography tank fittings.

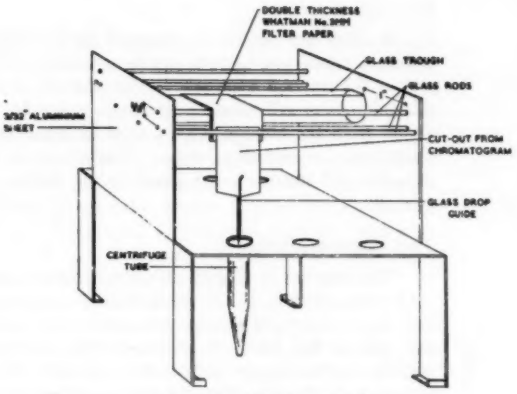

Fig. 2. Elution stand.

Chromatography tanks. Glass battery tanks 48 × 26 cm. were adapted by grinding flat the edges of the open end and fitting them with ground glass lids with a central hole to take a separating funnel. Two stainless steel frames, one on either side of the trough (Bush, personal communication), are held by a stand made to fit inside the tank (Fig. 1). The two narrower walls of the tank are lined with filter paper sheets, covering also the base of the tank. The filter paper is thoroughly moistened with the stationary phase and some, forming about a 5 cm. deep layer, poured into the tank. The mobile phase is then carefully poured in. The tanks are kept sealed with zinc oxide adhesive plaster.

The apparatus used for eluting the paper sections (*Fig.* 2). The stand was made from aluminium sheet, and glass rods were used to support the trough and papers. The interval between the edge of the trough and the upper supporting rod is about 2 cm., and the interval between the adjacent sides of the lower supporting rods should be 3 mm. Using these dimensions a wick consisting of a double thickness of Whatman 3 MM filter paper gives the desired flow of about 1·5 ml./hr. The illustrated glass clip to guide the drops into the tube is essential. The apparatus is covered with a glass jar to avoid evaporation.

Method.

Heparinized blood is collected into ice-cooled containers and centrifuged immediately. The plasma is either analysed within 24 hrs. or stored at — 15° C. Storage for a month does not produce any appreciable loss of steroid. It is convenient to analyse 6-8 samples at a time.

Analysis of 10 *ml. of plasma.*
Extraction.

The plasma is diluted with an equal amount of distilled water, and, if available, C^{14} steroids may be added to estimate recoveries. The extraction of steroids is carried out twice in a separating funnel with 50 ml. amounts of a mixture of ethylacetate-ether (2:1), shaking each for at least one minute. The combined extracts are washed for half a minute with 5 ml. of 0·75 N sodium hydroxide solution, followed by 5 ml. of a 0·5 p.c. acetic acid solution and two 5 ml. distilled water washings. The extract is quantitatively transferred to a double-necked, round-bottom flask and evaporated to dryness at 35° C. in a nitrogen atmosphere under reduced pressure.

Removal of fat.

A silica gel column is prepared by pouring a mixture of 1 gm. silica gel in approximately 5 ml. of ethylacetate-petroleum ether (1:1) into a 10 × 1 cm. chromatography column with a G2 sintered glass disc. The content of the flask is washed onto the column using 2 ml. volumes of ethylacetate-petroleum ether (1:1) to a total of 10-12 ml. The steroids are eluted from the column with a total of 7 ml. of ethylacetate-methanol (1:1), collecting the eluate into a centrifuge tube. The eluate is evaporated to dryness under a stream of nitrogen and steroids are washed to the bottom of the tube, with small portions of solvent mixture.

First chromatography.

The residue is quantitatively transferred inside a square 9 × 3 cm. on a 5 cm. wide paper strip (Fig. 1). The steroids are concentrated onto the upper line by repeated "running up" in a mixture of ethylacetate-methanol (2:1). A drop of sudan III is then applied to one end of this line. A reference strip is prepared by pipetting an ethanolic mixture of cortisol, corticosterone and cortisone (about 15 μg of each) onto a spot. The strips are arranged on frames (Fig. 1) and equilibrated overnight in a chromatography tank. The corticosteroids are separated by descending chromatography in toluene/75 p.c. methanol (Bush, 1952) at 30° C. for 2½ hrs.

After drying the strips at room temperature the individual steroids are located on the paper strip by their fluorescence in ultraviolet light with a peak emission of 2537 A.U. (Hanovia "Chromatolite"). The reference strip for detection of these areas is used if the concentrations in the analyzed sample are below 4 μg./cm. square. If a suitable ultraviolet source is not available the reference strip can be treated with blue tetrazolium and used as a guide. Sodium hydroxide treatment of the reference strip is not recommended as the spots run on when heated (see later). The areas are marked 2 cm. above and below the fluorescing spots and cut out with one end V-shaped. If the steroid is close to the line of the cut, it is necessary to move it towards the middle of the cut out section. This is done by placing the section on end on a filter paper, saturated with ethylacetate-methanol (2:1), in a suitable container, and allowing the solvent to ascend to the middle of the paper section. The process is performed twice on either end of the paper (Bush, personal communication).

Elution.

The cut out paper sections are eluted in the apparatus shown (Fig. 2) by using a mixture of ethylacetate-methanol (2:1). 2-3 ml. of eluate are collected for each fraction. Because aldosterone has a mobility only slightly faster than cortisol, it is present in the eluate from the cortisol section and is separated later. An aliquot is removed for the quantitative estimation of cortisol on a second chromatogram, the volume being selected so that the amount falls into the range of 0·5-3 μg. The remainder is used for aldosterone determination. If cortisol -4-C^{14} has been added a second aliquot is reserved for radioactivity measurement. From the corticosterone and cortisone eluates aliquots are removed only if radioactive measurements are to be made, or if the amounts are expected to be high.

Measurement of radioactivity.

The aliquots intended for radioactive measurements are transferred by washing into aluminium planchettes and evaporated on a hot plate at 40° C. Measurement of the radioactivity using a thin mica end-window G.M. tube and scaler gives a counting efficiency of about 4 p.c., i.e., approximately 600 counts/min./1 μg. steroid C^{14}. More efficient counting techniques, for example, windowless flow or scintillation counting, have the disadvantage of being time consuming and inconvenient.

Second chromatography.

The second chromatogram is performed on 2·5 cm. wide paper strips. The concentrated portions of the eluates reserved for cortisol, cortisone and corticosterone determination are transferred quantitatively to spots no more than 0·5 cm. diameter. This is achieved by repeated applications of very small amounts from fine calibre pipettes. Standards of cortisol, cortisone, and corticosterone in the range 0·3-5 μg. are applied to separate paper strips. The strips with unknowns and standards are arranged on the frames, equilibrated for 3 hrs. or overnight, and then run in toluene/75 p.c. methanol at 30° C. for 2¾ hrs.

The large remainder of the cortisol/aldosterone eluate is also applied on a spot. A reference strip containing cortisol, cortisone and aldosterone is prepared together with standards of preferably aldosterone, although cortisol may be used. The ends of these strips are cut into V-shape. They are run in toluene/75 p.c. methanol at 30° C. for 9¾ hrs.

When the time of the run was prolonged to 12 hrs. cortisone (if also present in the eluate) and aldosterone ran too close to each other for quantitative estimation. The individual steroids, when chromatographed, run the following distance from the starting line (the starting line being 5 cm. from the trough):—After 2¾ hrs.: cortisol 3·5-4 cm., aldosterone 4·2-4·5 cm., cortisone 8-8·5 cm., corticosterone 17·5-18 cm. After 9¾ hrs: cortisol 12-13 cm., aldosterone 17-18 cm., cortisone 20-21 cm., and corticosterone more than 34 cm., which is the total length of the paper.

Quantitative evaluation.

The strips are dried for 5 min. at room temperature. They are then dipped in a 2 N aqueous solution of sodium hydroxide in a shallow dish, so that they are drawn across the surface of the solution. They are then placed on glass plates, turning the ends round the edge of the plate and sticking them to the under surface with cellulose tape. They are dried in a horizontal position at 80° C. for 100 min. To avoid drifting of the spots as much as possible, it is essential to have an equal amount of moisture along the strip (just saturated with the sodium hydroxide solution) and to place it on the glass plate so that no air bubbles are left under it.

The concentrations are estimated by comparison of the yellow soda fluorescence in strong ultraviolet light with standard strips of similar concentration, using a medium pressure mercury arc as an ultraviolet light source. The found concentrations are corrected for losses as indicated by the recovery of added radioactivity and the quantity of added radioactive material is subtracted from the total.

Identification of the isolated fractions.

The nature of the corticosteroids isolated from the sheep's adrenal venous plasma by the described method was demonstrated by additional tests.

After the addition of cortisol -4-C^{14} and corticosterone -4-C^{14}, a large amount of pooled adrenal venous plasma was extracted and chromatographed as already described on two 5 cm. strips for 2¾ hrs. The strips were dried and one was used for colour reactions. It was cut into three strips, in the long axis. The blue tetrazolium reaction (Nowaczynsky, Goldner and Genest, 1955) was developed on the first, soda fluorescence on the second, and the Zimmerman reaction (Savard, 1953) on the third.

The other 5 cm. strip was divided into 5 cm. sections along its length. Each section was eluted and one-third removed from each eluate for acetylation. The rest of the eluates were transferred to 5 cm. paper strips and chromatographed for 6 hrs. Each 5 cm. strip was then cut into three in the long axis and treated for group reactions as described above. The aliquots removed were each evaporated and acetylated using 0·3 ml. of pyridine followed by 0·15 ml. of acetic anhydride. They were left overnight at room temperature and after evaporation applied to a spot on a 2·5 cm. strip. Authentic aldosterone was also acetylated

and applied to a spot. Aldosterone monoacetate (as supplied) was also spotted on a separate strip. The strips were then run in petroleum ether-benzene (2:1) -80 p.c. methanol (Bush, 1952) at 30° C. for 3 hrs. After drying, these strips were treated with sodium hydroxide and examined.

All the strips were examined with a thin mica end-window G.M. tube to locate radioactive areas. Reference compounds of cortisol, corticosterone, cortisone, aldosterone, 11-β-hydroxyandrostene-3:17-dione, 11-desoxycorticosterone and 11-dehydrocorticosterone were chromatographed and treated in the same way as the unknown compounds.

The colour reactions demonstrated that all the groups determining biological activity were present in the analysed substances, and the reactions carried out on each compound compared well with the reference substance of the same nature. The elution and second chromatography of the substances for 6 hrs. in the same system indicated that they were not artifacts because, with the exception of cortisol from which aldosterone separated, each substance remained as a well-defined single spot. Acetylation of the eluted fractions resulted in monoacetates of cortisol, cortisone and corticosterone and a diacetate of aldosterone. Radioactivity on the strips after chromatography was confined to the areas where the individual C^{14} steroids were located.

As a further check on the identity of the aldosterone-like spot separated by the described method, an extract of adrenal venous plasma was chromatographed in toluene/75 p.c. methanol at 30° C. for 9½ hrs. The aldosterone area was cut out, eluted and acetylated. Authentic aldosterone was acetylated and used as a reference. Chromatography showed the formation of a diacetate identical in mobility to the reference strip. Facilities were not available for a reliable bioassay of the material but, as shown in the Results section, the fact that this compound appears in the sheep's adrenal venous blood only during sodium depletion lends further support to the identification.

RESULTS.

Well-defined spots are essential for accurate quantitative evaluation. It was noticed, for example, that if the cortisol/aldosterone eluates contained a large amount of cortisol (more than 30 μg.) the aldosterone area in the second chromatogram became diffuse, and it was necessary to elute and run it as a spot again for 2½ hrs. The optimum amounts are between 0·5-3 μg. of substance in a 1·5 cm. square spot. Quantities up to 5 μg. could be estimated but above this concentration the results were inaccurate. In the author's experience, visual comparison by a number of observers working independently yielded results which differed by no more than 10 p.c. The precision was increased by using a large range of standards, and viewing the fluorescing areas through a yellow filter. This method for the quantitative estimation of soda fluorescence is quite satisfactory, although probably greater precision could be obtained by the photometric instrument devised by Ayres, Garrod, Simpson and Tait (1957). We found the other colorimetric and fluorimetric methods such as the micro blue tetrazolium method described by Vogt (1955) and potassium butoxide fluorescence method described by Abelson and Bondy (1955) subject to serious interference by variable paper blank readings, and of a low sensitivity for concentrations below 2 μg.

TABLE 1.

Recovery experiments.

(a) *Pure steroids added to water and extracted as plasma.*

Nature of added steroid	No. of determinations	Amount added (μg)	Percentage recovery	
			Chemical (range)	Radioactive
Non-radioactive cortisol	4	45 10	88-97	
Non-radioactive corticosterone	4	2	89-100	
Non-radioactive cortisone	2	5	90, 93	
Non-radioactive aldosterone	1	0·5	88	
Cortisol-4-C^{14}	2	8		94, 96
Corticosterone-4-C^{14}	2	2		93, 97

(b) *Pure non-radioactive steroids added to* 10-15 *ml. sheep adrenal venous blood plasma before extraction.*

Nature of added steroid	No. of determinations	Amount added (μg)	Percentage chemical recovery	
			Range	Mean
Cortisol	4	10	84-96	90
Corticosterone	4	3	86-93	89
Cortisone	4	5	88-97	92
Aldosterone	2	0·5 2·0	76 87	

(c) *Pure radioactive steroids added to* 10-15 *ml. sheep adrenal venous blood plasma before extraction.*

Nature of added steroid	No. of determinations	Amount added (μg)	Percentage radioactive recovery	
			Range	Mean
Cortisol-4-C^{14}	30 10	9 4	88-96 90-97	93 92
Corticosterone-4-C^{14}	25	2	89-95	93

The mean recovery values of added radioactivity from 65 plasma samples averaged 92 p.c. (Table 1 c). The recovery of aldosterone was assumed to be the same as of cortisol -4-C^{14}. No radioactivity was lost in the transfer of the eluates to the paper. When pure non-radioactive cortisol, cortisone, corticosterone and aldosterone were added to adrenal venous plasma, the mean recovery values at the very end of the procedure averaged 90 p.c. and were very similar for the individual steroids (Table 1b). This agreed fairly well with the radioactivity recoveries. When pure steroids were added to water and extracted by the same procedure recoveries were only a little higher than those

from plasma (Table 1a). The results indicate that only about 5-10 p.c. is lost during the manipulation procedures. The considerable losses which usually take place during the elution processes are eliminated by using the simple device described, which ensures total recoveries. Duplicate analyses agreed within a range of ± 5 p.c.

Range of cortisol and cortisone concentrations in peripheral blood plasma of normal human subjects.

Plasma samples were obtained by venipuncture from 6 normal males aged between 19-36 years. All bleedings were taken at about 4 p.m. 10-13 ml. of plasma were analysed by this method. The concentrations ranged:—cortisol, 7-13 μg./100 ml. plasma; cortisone, 1·5-5 μg./100 ml. plasma. In one sample where cortisol was 23 μg./100 ml. plasma, the high concentration may have been due to the fact that this person was in a highly excited state before the time of bleeding. When plasma was obtained from this person on another occasion the cortisol concentration was 8·5 μg./100 ml. plasma.

The results obtained are similar to those observed in normal subjects by others using chromatographic techniques. Sweat (1955) obtained a range for cortisol of 10·8 ± 2·6 μg./100 ml. of plasma, Morris and Williams (1953) obtained for cortisol a range of 6·5-10·5 μg./100 ml. plasma, Bondy and Upton (1957) obtained a range for cortisol of 4-13 μg./100 ml. plasma and for corticosterone 0·05 μg./100 ml. plasma. Ayres, Garrod, Pearlman, Tait and Tait (1957) give a range for cortisol as high as 5-27 μg./100 ml. of plasma and for corticosterone 0·3-0·6 μg./100 ml. plasma.

The range of estimated steroids in patients with suspected adrenocortical disorders.

Plasma was obtained by venipuncture from a patient with a suspected adrenal tumor. The concentrations of the estimated steroids were: cortisol 8·2 μg, cortisone 2·3 μg., and corticosterone 0·6 μg. per 100 ml. plasma. By laparotomy no tumor was discovered.

Two patients with suspected Cushing's syndrome with hirsutism were studied before and during the intravenous infusion of 25 I.U. of A.C.T.H. (Corticotrophin, C.S.L.) over a period of 6 hrs. Before infusion the concentrations per 100 ml. plasma were: cortisol, 6·3 μg.; cortisone, 1·5 μg.; corticosterone, none detected in 15 ml. plasma; and cortisol, 18·5 μg.; cortisone, 8·2 μg., and corticosterone, 1·5 μg. After 5¼ hrs. infusion the concentrations/100 ml. plasma had risen respectively to: cortisol, 44·4 μg.; corticosterone, 5·1 μg.; and cortisol, 40·4 μg. and corticosterone, 4·2 μg. Cortisone fell in both patients to nearly half the initial value being respectively 0·9 μg. and 4·2 μg. per 100 ml. plasma. The patient with the initially high corticosteroid level had the more definite clinical picture of the disease.

The concentrations of estimated steroids in the adrenal venous blood plasma of sheep.

On a number of occasions adrenal venous blood was collected from two conscious undisturbed sheep with transplanted adrenal glands (McDonald, Goding and Wright, 1958) when in normal sodium balance. A total of ten plasma samples were analysed. The range of concentrations of the analysed steroids per 15 ml. plasma was: cortisol 15-30 μg, corticosterone 0-1·2 μg., aldosterone none detected in 75 ml. of plasma. The range of secretion rate/hr. was: cortisol 150-600 μg., corticosterone (< 1)-10 μg.

Adrenal venous blood was collected from the same animals in a conscious state at various stages of sodium depletion. A total of 10 plasma samples analysed on different occasions contained concentrations per 15 ml. plasma: cortisol 20-76 μg., corticosterone 0·1-2·1 μg., aldosterone 0·2-1·7 μg. The range of secretion rate/hr. was: cortisol 180-940 μg., corticosterone 2-32 μg., aldosterone 7-23 μg.

DISCUSSION.

The technique described has been used for more than twelve months in this laboratory and about 100 plasma samples were analysed. In our experience, provided certain procedures are carefully carried out, it is reliable and convenient. Losses due to manipulations did not exceed 10 p.c. If other losses occurred they were due to breakdown of steroids caused by oxidation, long exposure to ultraviolet light, or heat. We found it essential to purify and freshly distil all solvents before use. All evaporations were carried out at temperatures below 40° C. Using a special pump, 25-30 ml. of solvent were evaporated per minute. If it was necessary to interrupt the analysis, the extracts were stored in a dry state at a temperature − 15° C. In the author's experience, three days was the maximum time of storage of the eluted individual steroid fractions as, when these minute quantities were stored for longer periods, losses occurred. The solvent mixture used for the extraction of steroids from plasma is a very convenient one, as no emulsions have been noticed when used in the given proportions. Two extractions of the sample are enough to extract all the free or weakly bound steroids from the plasma. On extracting it for the third time no steroids were detected in a plasma sample containing more than 100 μg. of extractable steroids. The removal of fat was effected by a number of procedures described by Bush as the reverse phase "defatting" chromatography, and the partitioning in 70 p.c. ethanol (Bush and Ferguson, 1953). The purest product was obtained with the highest recovery by using the silica gel column for the fat separation. The most important factor in Bush's chromatography system is the temperature. 30° C. seemed a very convenient temperature for all our runs. The tendency for condensation (and

therefore uneven runs) was greatly diminished by fitting out the tanks as described which kept the atmosphere in the tank very uniform. The soda fluorescence used for quantitative estimation of individual steroids was found to be a very easy and convenient procedure. The eluted steroid being rechromatographed becomes purer and, provided the spots are well defined, this procedure is relatively accurate, considering the lower sensitivity and interference of high blank readings in colorimetric and spectrophotometric methods.

The appearance of aldosterone in the small amounts of plasma of the sodium-depleted sheep only, and the rise in plasma cortisol and corticosterone in humans after A.C.T.H. administration, supported by the qualitative studies on pooled adrenal venous plasma of the sodium-depleted sheep, suggest that the technique is of adequate specificity.

At the beginning of this work a substance was often noticed in the chromatograms of plasma extracts which had a similar mobility to 11-β-hydroxyandrost-4-ene-3:17-dione (11-hydroxyandrostene-dione) in toluene 75 p.c./methanol. After the addition of cortisol -4-C^{14} to the plasma before extraction this substance was found to be radioactive, and therefore a breakdown product of cortisol. It was usually of the order of 3-5 p.c. of total cortisol. The substance gave a positive sodium hydroxide fluorescence test, but did not reduce blue tetrazolium and gave only a slightly positive Zimmerman reaction. By a number of experiments it was found that the breakdown of cortisol took place during evaporation, and was due to small amounts of alkali left in the extract. By washing the extract with acetic acid and making the evaporation more efficient it was eliminated completely. A substance probably similar to the one mentioned was found by Reif and Longwell (1958) who assumed it to be an oxidation product of cortisol.

Extractable substances worth mentioning were noticed in extracts of haemolyzed human and sheep plasma samples. When chromatographed one ran between corticosterone and 11-hydroxyandrostene-dione. This substance, when viewed in ultraviolet light at 2537 A.U., gave an intense blue fluorescence similar to that given by steroids. It gave negative tests for both sodium hydroxide fluorescence and blue tetrazolium. The other substance had a mobility just slightly faster than 11-hydroxyandrostene-dione. It did not fluoresce when in ultraviolet light, but gave a delayed positive reaction with blue tetrazolium and a white fluorescence in strong ultraviolet light after sodium hydroxide treatment. These substances might be a cause of high blank readings if used in some colorimetric or spectrophotometric determinations of steroids.

The described method was found to be specific and accurate, with an average recovery of 92 p.c. for the individual steroids. If radioactive steroids can be added to the analysed sample a correction for the incomplete chemical recovery can be applied and absolute amounts can be calculated to an accuracy $\pm$ 10 p.c. The method has been successfully applied to the analysis of human

peripheral blood plasma as well as the peripheral and adrenal venous blood plasma of conscious sheep. The technique is very suitable for serial analysis of small volumes of peripheral blood plasma in tests for adrenal function in clinical medicine. The results of the investigations on sheep will be presented in forthcoming publications.

SUMMARY.

Some modifications of the Bush chromatography procedures have been devised which permit an average of 92 p.c. recovery of the corticosteroids present in plasma samples. A single solvent system is used for chromatographic separation of steroids after previous purification of the extract. Quantitative estimation has been made of cortisol, corticosterone, cortisone and aldosterone, using a sodium fluorescence method with an accuracy of ± 10 p.c. If radioactive steroids are added, correction of the incomplete chemical recovery can be applied for each sample analysed. A detailed description of the method and identification of isolated corticosteroid fractions, with diagrams of the apparatus involved, is presented.

The concentration range of estimated steroids in peripheral blood plasma of normal human subjects was: cortisol 7-13 μg., cortisone 1·5-5 μg. per 100 ml. plasma. In two patients with suspected Cushing's syndrome with hirsutism the concentrations were: cortisol 6·3 μg., cortisone 1·5 μg. per 100 ml. plasma; and cortisol 18·5 μg., cortisone 8·2 μg., and corticosterone 1·5 μg. per 100 ml. plasma respectively. After A.C.T.H. infusion the concentrations were respectively: cortisol 44·4 μg., cortisone 0·9 μg., corticosterone 5·1 μg. per 100 ml. plasma; and cortisol 40·4 μg., cortisone 4·2 μg., corticosterone 4·2 μg. per 100 ml. plasma.

The secretion rate of estimated steroids in adrenal venous blood plasma of conscious undisturbed sheep with a transplanted adrenal gland was in the range: cortisol 150-600 μg. per hr., corticosterone (< 1)-10 μg. per hr. The adrenal secretion of the same animals when analysed at various stages of sodium depletion was: cortisol 180-940 μg. per hr., corticosterone 2-32 μg. per hr., aldosterone 7-23 μg. per hr.

Problems involved in the chromatographic procedures of steroid analysis are discussed.

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REFERENCES.

Abelson, D., and Bondy, P. K. (1955): Arch. Biochem. Biophys., 57, p. 208.

Ayres, P. J., Garrod, O., Pearlman, W. H., Tait, J. F., Tait, S. A. S., and Walker, G. (1957): Ciba Foundation Colloquia on Endocrinology, 11 (Churchill: London), p. 309.

Ayres, P. J., Garrod, O., Simpson, Sylvia A., and Tait, J. F. (1957): Biochem. J., 65, p. 639.
Ayres, P. J., Simpson, Sylvia A., and Tait, J. F. (1957): *Ibid.*, 65, p. 647.
Bondy, P. K., and Upton, Virginia (1957): Proc. Soc. exp. Biol. Med., 94, p. 585.
Borth, R. (1956): Acta Endocrinol., 22, p. 125.
Bush, I. E. (1952): Biochem. J., 50, p. 370.
Bush, I. E. (1952-53): J. Endocrinol., 9, p. 95.
Bush, I. E., and Ferguson, K. A. (1953): *Ibid.*, 10, p. 1.
Bush, I. E., and Sandberg, A. A. (1953): J. biol. Chem., 205, p. 783.
Bush, I. E. (1954): Rec. Prog. Hormone Res., 9, p. 321.
Eberlein, W. R., and Bongiovanni, A. M. (1955): Arch. Biochem., 59, p. 90.
Garrod, O., Simpson, Sylvia A., and Tait, J. F. (1956): Proc. Roy. Soc. Med., 49, p. 885.
McDonald, I. R., Goding, J. R., and Wright, R. D. (1958): Austral. J. exp. Biol., 36, p. 83.
Morris, C. J., and Williams, D. C. (1953): Biochem. J., 54, p. 470.
Neher, R., and Wettstein, A. (1956): J. clin. Invest., 35, p. 800.
Nowaczynsky, W., Goldner, M., and Genest, J. (1955): J. lab. clin. Med., 45, p. 818.
Reif, A. E., and Longwell, B. B. (1958): Endocrinology, 62, p. 573.
Savard, K. (1953): J. biol. Chem., 202, p. 459.
Sweat, M. L. (1955): J. clin. Endocrin. Metab., 15, p. 1043.
Vogt, Marthe (1955): J. Physiol., 130, p. 601.
Zaffaroni, A. (1953): Rec. Prog. Hormone Res., 8, p. 51.

THE ACTION OF RESERPINE ON THE ISOLATED HEART

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Although reserpine is used to reduce hypertension, several workers have recently reported transient pressor responses immediately following the intravenous administration of this drug.

Thus Domino and Rech (1957) noted that reserpine acetate or phosphate given intravenously produced 27 p.c. increase in the femoral arterial pressure of unanaesthetized dogs; Maxwell *et al.* (1957) recorded similar pressor responses in both anaesthetized and spinal dogs, and suggested a possible direct peripheral action involving the sympathomimetic humoral mechanism. Krayer and Paasonen (1957) observed that tachycardia was associated with the reserpine-induced positive inotropic response they recorded in the dog heart-lung preparation.

Recent literature suggests that reserpine liberates nor-adrenaline and possibly adrenaline from various tissues (Burn and Rand, 1957; Krayer and Paasonen, 1957; Muscholl and Vogt, 1957) and it seemed possible that the pressor responses recorded by the above workers were due to the liberation of these sympathomimetic amines which would then be free to produce their pharmacological effects, i.e. pressor activity, associated with tachycardia in the isolated heart. This present investigation was carried out to determine whether reserpine produced any direct effect upon the isolated spontaneously beating heart and to compare the response with that produced by the amines under identical conditions, metabolic as well as physiological effects being recorded.

Method.

The isolated spontaneously beating hearts of unselected summer male and female toads of species *Bufo marinus* were used throughout this study, perfusions being carried out at $25{\cdot}0 \pm 0{\cdot}05°$ C. with glucose as the sole exogenous substrate. The perfusion apparatus was used as previously described (Nayler and McKelvie, 1956) for the simultaneous recording of the beat rate, drops per beat, maximum aortic pressure, rate of oxygen utilization and carbon dioxide output of the isolated heart. Two E.C.G. leads introduced into the vessel were attached one to the ventricular apex and the other to the left aortic arch by saline-soaked cotton ties so that E.C.G. records could be taken directly. The apparatus was oxygenated for ten minutes and a further thirty minutes allowed for temperature equilibration at $25{\cdot}0°$ C. before observations were commenced.

[1] The expenses of this investigation were defrayed by a grant from the Life Insurance Medical Research Fund of Australia and New Zealand.

In six preparations following an initial one hour's control perfusion during which time observations were recorded at fifteen-minute intervals, reserpine[2] was added to the perfusion fluid to give a final concentration of 1 μg/ml. Records of beat rate, drops per beat, maximum aortic pressure, rate of oxygen uptake and carbon dioxide output were continued at fifteen-minute intervals throughout the following two hours. In several of these preparations E.C.G. records were taken regularly, together with the above observations prior to and following the addition of the reserpine to the perfusate.

In another series of six experiments Regitine[3] was initially added to the perfusate to give a final concentration of 1 μg./ml. After the standard one hour's perfusion reserpine was added to the Regitine-enriched perfusate as above and observations continued throughout the subsequent two hours.

The positive inotropic effect of reserpine in the above concentration was also recorded in a series of six standard Straub recycling toad heart preparations. Kymograph recordings of the force of ventricular contraction were used to measure the response.

The results from these experiments have been compared with those from an earlier series in which nor-adrenaline and adrenaline were similarly added to a series of perfused hearts to give final concentrations of 0·1 μg./ml. and 0·01 μg./ml. respectively (Nayler, 1956).

RESULTS.

The results of four-hour control perfusions at 25·0° C. have been described previously (Nayler, 1956b). Only small fluctuations in respiratory quotients and rate of oxygen utilization were recorded, together with a slow steady decline in maximum aortic pressure and drops per beat.

The action of reserpine (1 μg./ml.) on the series of isolated hearts is summarised in Figs. 1-3, where in each case the mean responses noted in six experiments are compared with those recorded following the administration of adrenaline (0·1 μg./ml.) and nor-adrenaline (0·1 μg./ml.) to a similar series. Reserpine when added at this concentration to hearts previously perfused in standard Ringer solution always evoked an increase in aortic pressure evident throughout the following ninety minutes. In Fig. 1 it is apparent that the time interval during which the reserpine-induced inotropic response was recorded exceeded that occupied by the pressor response due to a single dose of either amine. Regitine (1 μg./ml.) completely blocked the pressor activity of reserpine in this preparation. The positive inotropic activity of reserpine was similarly observed in those preparations using kymograph recordings of the force of ventricular contraction as the index of response. In Fig. 4 the results of two such preparations in which reserpine was added at the above concentration are shown, and it will be noted that the increased force of contraction evident ten minutes after the addition of the drug to recycling hearts at room temperature followed a similar time course to that of the increased aortic pressures recorded in Fig. 1.

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[2] Reserpine as Serpasil : Ciba product.
[3] Regitine: Ciba product. (Phentolamine, U.S.P.)

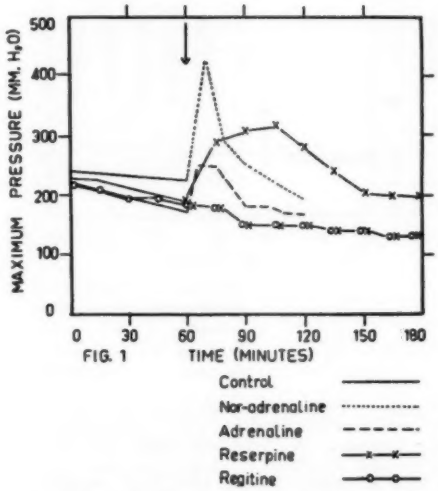

Fig. 1. Graph showing effect of adrenaline (0·01 microgram/ml.) nor-adrenaline (0·1 microgram/ml.), reserpine (1 microgram/ml.) and reserpine plus Regitine (1 microgram/ml.) on the aortic pressures of a series of perfused hearts. In each case the mean results of six experiments are displayed.

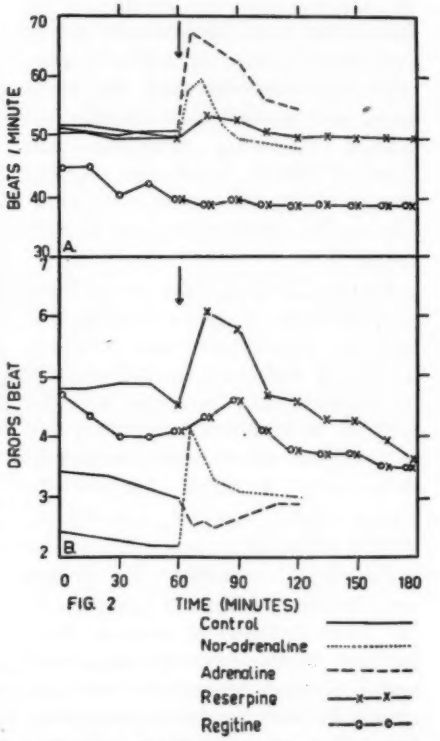

Fig. 2. Effect of adrenaline (0·01 microgram/ml.), nor-adrenaline (0·1 microgram/ml.), reserpine (1 microgram/ml.) and reserpine plus Regitine (1 microgram/ml.) on the beat rate (2A) and drops per beat (2B) of the perfused heart. Each curve represents the mean results of six experiments.

Coincident with its pressor activity reserpine consistently induced slight tachycardia which was evident through the following hour (Fig. 2A). This tachycardia following reserpine treatment was associated with increased stroke volume (as indicated by the increased number of drops per beat) similar to that noted following nor-adrenaline administration. By contrast adrenaline caused an actual fall in the stroke volume (Fig. 2B).

As was previously noted with adrenaline and nor-adrenaline the positive inotropic action of reserpine was always associated with increased oxidative metabolism and slightly raised respiratory quotients (Figs. 3A, B). Comparison between Figs. 1 and 3A, B reveals that the increased oxidative metabolism due

to both reserpine and the amines followed the same time course as the respective positive inotropic response. This correlation between the metabolic and physiological changes recorded following reserpine and catechol amine treatment suggests the possibility that some fundamentally similar action is involved in both cases.

In Figs. 1, 2 and 3 it is apparent that Regitine (1 μg./ml.) suppressed both the physiological and metabolic actions of reserpine, the tachycardia, augmented aortic pressures and the increase in oxidative metabolism due to reserpine alone being completely suppressed. A slight increase in stroke volume, however, was still recorded (Fig. 2B).

The E.C.G. records taken before and after the addition of reserpine to the heart perfused at constant temperature in the perfusion apparatus reveal changes typical of those recorded by other workers investigating the action of adrenaline and noradrenaline on isolated frog hearts (Fig. 5). This response is characterized by the augmented amplitude of the 'Q R S' complex and increased negativity of the 'T' wave (Baker and Baker, 1955).

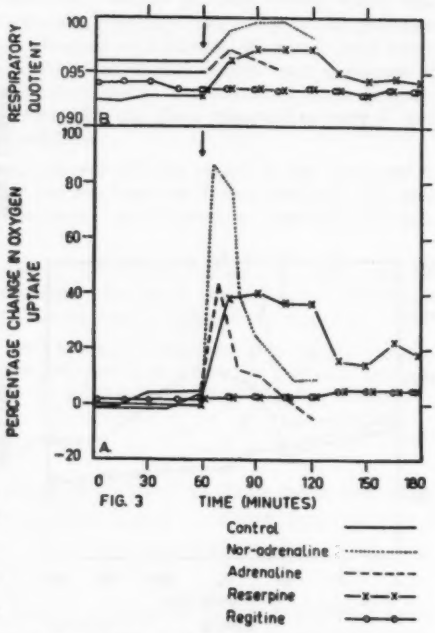

FIG. 3

Fig. 3. Effect of adrenaline (0·01 microgram/ml.), nor-adrenaline (0·1 microgram/ml.), reserpine (1 microgram/ml.) and reserpine plus Regitine (1 microgram/ml.) on the rate of oxygen uptake (3A) and respiratory quotients of the isolated hearts. In each case the mean results of six experiments are shown.

DISCUSSION.

These results show that reserpine at a concentration of 1 μg./ml. consistently exerted a positive inotropic effect on the isolated toad heart used in this preparation, and that this response is physiologically and metabolically comparable with that of the sympathomimetic amines.

Whereas Barrett *et al.* (1954) failed to detect any direct peripheral effect due to reserpine and so concluded that the observed clinical effects of hypotension and bradycardia were of central origin, later investigators recorded a transient pressor response preceding the hypotensive effects in intact animal studies. Domino and Rech (1957) using unanaesthetized dogs paralyzed with

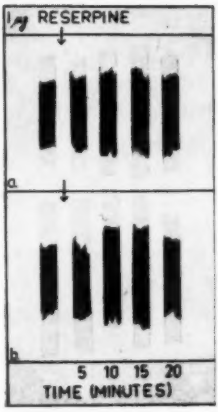

Fig. 4. Kymograph tracings of two recycling hearts showing positive inotropic effect of 1 microgram of reserpine added at the arrow. Note that the effect is still evident after fifteen minutes.

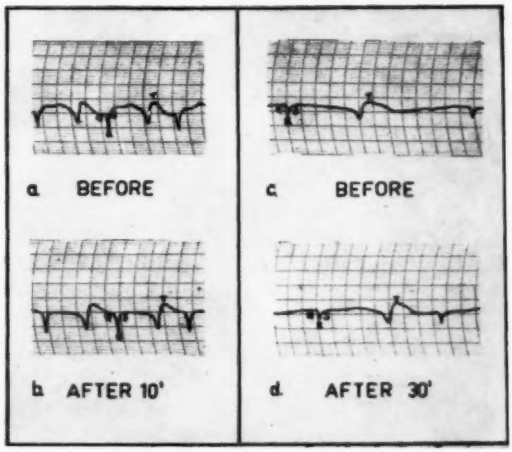

Fig. 5. E.C.G. tracings from two perfused hearts.
5 a, b: Immediately before and 10 minutes following addition of 1 microgram of reserpine.
5 c, d: Immediately before and 30 minutes following addition of 1 microgram of reserpine.
Note increased 'Q R S' complex and increased negativity of the 'T' wave.

gallamine or decamethonium noted a rise in arterial pressure within 5 to 20 minutes following the intravenous administration of large doses of reserpine acetate or phosphate. They found that bilateral adrenalectomy did not modify the pressor response and that ganglionic blockade enhanced it.

Although it has been established that reserpine does release bound serotonin from tissues (Pletscher *et al.*, 1955; Shore *et al.*, 1956), the release of serotonin is apparently not involved in the pressor activity of reserpine since large doses of cocaine (Domino and Rech, 1957) or L.S.D. (Maxwell *et al.*, 1957) failed to suppress the positive inotropic effect. The last workers suggested that this pressor activity in the intact animal was due to the release of some sympathomimetic agent from the adrenal medullae or other sites, to a direct peripheral action of reserpine in blood vessels, or to a ganglionic stimulant action.

Our results indicate that the pressor response of reserpine detected by these workers using intact animals is due, in part at least, to a direct cardiac action.

Recent work has shown that reserpine releases the catechol amines as well as serotonin from tissues. Burn and Rand (1957) found reduced amine content of rabbit aortae following reserpine therapy, the mean concentration being diminished from 0·47 to 0·11 μg./gram. These workers (1958) also noted that

aortic strips from reserpine-treated rabbits were much more sensitive to nor-adrenaline and adrenaline than were normal strips. Krayer and Paasonen (1957) recorded a marked decrease in the amine content of right auricle and left ventricle of the dog heart after three hours exposure to therapeutic doses of the drug and the complete absence of nor-adrenaline from the hearts of dogs previously treated for three days with reserpine. This depletion of the amine content of heart following reserpine therapy has been confirmed by Bertler *et al.* (1957) and Innes and Krayer (1957).

Although the origin of the catechol amines present in various tissues including the myocardium and arterial walls is not known for certain, there is some evidence to support the assumption that a steady supply from sympathetic nerve fibres is involved (von Euler, 1946; Goodall, 1951). Burn and Rand (1958) have suggested that a small leakage from this store may occur normally and that this may play some part in determining muscular tone.

Since the pressor response of reserpine recorded in our experiments was completely abolished by Regitine at a concentration sufficient to block the adrenergic action of the amines (de V. Cotten *et al.*, 1957) and because of the striking similarity between the pressor responses due to the sympathomimetic amines and reserpine, the involvement of these amines in this reserpine pressor activity is possible.

Hence the positive inotropic action of reserpine on the isolated heart and the associated tachycardia, changed E.C.G. pattern, raised level of oxidative metabolism and respiratory quotients, are probably due to the release of catechol amines from the heart into the circulatory system. Before these amines are destroyed by the amino oxidase systems they would be free to produce the described physiological and metabolic effects. Since the response recorded in these experiments was continued over sixty minutes it is likely that the amines are released gradually rather than in one short burst. Alternatively, the amine oxidase system may be suppressed by reserpine so that the free circulating amines are not rapidly destroyed.

SUMMARY.

Reserpine (1μg./ml.) produced a transient positive inotropic effect on the isolated spontaneously beating toad heart.

The inotropic effect was associated with tachycardia, increased oxidative metabolism and raised respiratory quotients.

The response was suppressed by the presence of Regitine (1 μg./ml.).

E.C.G. changes recorded immediately following the addition of reserpine are similar to those due to the catechol amines.

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REFERENCES.

Baker, W. M., and Baker, Judith (1955): J. Pharmacol. exp. Therap., 113, p. 132.
Barrett, W. E., Rutledge, R. A., and Rogie, B. (1954): Fed. Proc., 13, p. 334.
Bertler, A., Carlsson, A., Nilsson, J., and Rosengren, E. (1957): Acta physiol. Scand., 42, Suppl. 145, p. 22.
Burn, J. H., and Rand, M. J. (1957): Lancet, 2, p. 1097.
Burn, J. H., and Rand, M. J. (1958): Brit. med. J., 1, p. 903.
De V. Cotten, Marion, Moran, N. C., and Stopp, Phyllis E. (1957): J. Pharmacol. exp. Therap., 121, p. 183.
Domino, E. F., and Rech, R. H. (1957): *Ibid.*, 119, p. 142.
Von Euler, U. S. (1946): Acta physiol. Scand., 11, p. 168.
Goodall, McC. (1951): *Ibid.*, 24, Suppl. 85.
Innes, I. R., and Krayer, O. (1957): J. Physiol., 139, p. 18.
Krayer, O., and Paasonen, M. K. (1957): Acta physiol. Scand., 42, Suppl. 145, p. 88.
Maxwell, R. A., Ross, S. O., Plummer, A. J., Siggs, E. B. (1951): J. Pharmacol. exp. Therap., 119, p. 69.
Muscholl, E., and Vogt, M. (1957): Brit. J. Pharmacol., 12, p. 532.
Nayler, Winifred G., and McKelvie, D. (1956): Austral. J. exp. Biol., 34, p. 239.
Nayler, W. G. (1956): *Ibid.*, 34, p. 247.
Pletscher, A., Shore, P. A., and Brodie, B. B. (1955): Science, 122, p. 374.
Shore, P. A., Pletscher, A., and Brodie, B. B. (1956): J. Pharmacol exp. Therap., 116, p. 51.

THE POSSIBLE CLINICAL SIGNIFICANCE OF ELEVATIONS OF THE SERUM MUCOPROTEIN AND HEXOSAMINE FRACTIONS IN HUMAN DISEASE[1]

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Elevations of either the serum mucoprotein or hexosamine levels have been reported in a variety of diseases including malignancy, diabetes mellitus, hypothyroidism, experimental hypothyroidism, rheumatic arthritis, disseminated lupus erythematosus, and atherosclerosis. The significance of elevations of the levels of these substances has, however, remained uncertain, although such changes have been considered by many to reflect a breakdown or depolymerization of mucopolysaccharides of the connective tissue ground substance. Experimental evidence for such an assumption has been lacking.

In this paper, the results of the effects of the depolymerizing enzyme, testicular hyaluronidase, on the serum mucoprotein and hexosamine levels of the rat are reported, together with the serum levels of these fractions in male patients with acute rheumatic fever, acute nephritis, nephrosis, diabetes mellitus, dermatomyositis and scleroderma. The possible clinical significance of such changes is discussed.

METHODS.

Twenty Wistar rats of approximately six months of age were paired according to litter, weight, and sex and then randomly divided into two equal groups, test and control. The animals of the test group received a daily intramuscular injection of 300 turbidity reducing units of testicular hyaluronidase[3] contained in 0·5 ml. of normal saline, while the controls received 0·5 ml. saline alone. Injections were continued for 21 days when blood samples were collected by intra-cardiac puncture under ether anaesthesia. The rats received a normal basic diet as described by Johnson *et al.* (1958).

Single venous blood samples were collected from the median cubital veins of 23 male diabetic patients controlled by insulin treatment, 11 male patients with acute rheumatism, 5 patients with acute nephritis (Ellis type I), 2 patients with nephrosis (Ellis type II), and one patient with acute rheumatic chorea, one with scleroderma, and one with dermatomyo-

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sitis. Sera were obtained in all cases by centrifugation at 3,000 r.p.m. for 10 minutes. All determinations were performed in duplicate, and without any knowledge of the diagnosis of patients under consideration.

Serum mucoprotein was determined using a modification of the original method of Winzler *et al.* (1948) using 0·2 ml. of serum. The protein content of the mucoprotein was determined by the sensitive method of Lowry *et al.* (1951), and mucoprotein was then expressed as mg./100 ml. of serum using human albumin as a standard.

Serum hexosamine.—This fraction was determined using a modification of the method of West and Clarke (1938). Both methods have been described in more detail in a previous paper (Schwartz and Gilmore, 1958).

RESULTS.

Administration of testicular hyaluronidase produced elevations of both the serum mucoprotein and hexosamine levels (Table 1). The mean levels in the control group were 180·7 and 139·6 mg./100 ml. respectively, and in the treated rats the mean levels were 357·1 and 165·2 mg./100 ml. These elevations due to the action of testicular hyaluronidase are both highly significant ($p < 0·001$).

The data from the human subjects are collected in Table 2. Elevations of both the serum mucoprotein and hexosamine levels above the values for normal male subjects of comparable age groups were found in over one-half of the insulin-controlled diabetic subjects. Some of the highest values obtained were in those patients whose condition was complicated by degenerative vascular disease, but elevations were not restricted to patients with such complications.

Considerable elevations of both the serum mucoprotein and hexosamine levels were noted in all cases of acute rheumatic fever. These elevations were

TABLE 1.

The effect of Hyaluronidase on the serum mucoprotein and hexosamine levels in the normal-fed rat.

Serum mucoprotein (mg./100 ml.)		Serum hexosamine (mg./100 ml.)	
Hyaluronidase group	Control group	Hyaluronidase group	Control group
388	167	184	166
294	196	174	150
392	219	176	146
362	165	165	141
346	165	167	149
366	165	155	140
339	133	145	125
389	176	171	137
345	188	151	133
350	233	164	109
357·1	180·7	165·2	139·6

TABLE 2.

The serum mucoprotein and hexosamine levels in insulin-treated diabetic males, males with acute rheumatism, acute nephritis and miscellaneous disorders.

Condition	Age (years)	Serum mucoprotein (mg./100 ml.)	Serum hexosamine (mg./100 ml.)
Diabetes mellitus	17	130*	121
Diabetes mellitus	17	118	—
Diabetes mellitus	26	96	103
Diabetes mellitus	29	118	106
Diabetes mellitus	30	123	103
Diabetes mellitus	41	105	—
Diabetes mellitus	44	79	—
Diabetes mellitus	46	94	105
Diabetes mellitus	48	118	—
Diabetes mellitus	49	89	—
Diabetes mellitus	50	145	125
Diabetes mellitus	51	105	114
Diabetes mellitus	52	174*	135
Diabetes mellitus	58	98	106
Diabetes mellitus	64	84	—
Diabetes mellitus	65	115	101
Diabetes mellitus	67	84	99
Diabetes mellitus	68	183*	139
Diabetes mellitus	73	75	—
Diabetes mellitus	74	100	90
Diabetes mellitus	76	87	—
Diabetes mellitus	78	105	—
Diabetes mellitus	79	106	117
Acute rheumatism	14	160	123
Acute rheumatism	15	125	122
Acute rheumatism	17	160	124
Acute rheumatism	17	138	111
Acute rheumatism	17	158	139
Acute rheumatism	20	170	134
Acute rheumatism	20	306	187
Acute rheumatism	30	162	140
Acute rheumatism	32	248	147
Acute rheumatism	39	119	109
Acute rheumatism	44	216	140
Acute nephritis	36	115	100
Acute nephritis	37	273	156
Acute nephritis	37	112	114
Acute nephritis	37	242	108
Acute nephritis	42	175	122
Nephrosis	46	100	101
Nephrosis	48	163	122
Rheumatic chorea	14	103	86
Scleroderma	40	143	117
Dermatomyositis	40	131	117
Normal Males (n† = 24) (Mean Age 25)		86·6(±2·61)	92·2(±1·33)
Normal Males (n† = 16) (Mean Age 52)		95·6(±2·81)	97·1(±1·33)

* Complicated by ischaemic heart disease.
† Number of subjects investigated.
Estimated standard error of the mean in parentheses.

of a greater degree and more consistent than those found in patients with diabetes mellitus. Elevations of both fractions were also noted in the 5 cases of acute nephritis, and in one case of nephrosis, despite a marked hypoproteinaemia in the latter. The mucoprotein level was but slightly elevated in one case of rheumatic chorea, while the hexosamine value was normal. Definite elevations of both fractions were obtained in the cases of dermatomyositis and scleroderma.

DISCUSSION.

Hyaluronidase is regarded as an enzyme capable of chondroitinase and hexosaminidase activity (Gersh and Catchpole, 1949; Meyer, 1954) and its effect in causing elevations of the serum hexosamine and mucoprotein levels in the rat suggests that the levels of these substances in the serum may be taken as an index of the degree of depolymerization of connective tissue glycoprotein. A similar elevation of the serum hexosamine level has been shown in cholesterol fed rabbits after prolonged hyaluronidase treatment (Schwartz *et al.*, 1958).

Elevations of these fractions in certain diseases such as those described, may reflect a fundamental change in the connective tissue ground substance, and it would appear that such a change is a common factor in a number of otherwise unrelated diseases. Infiltration of tissue by malignant cells, tuberculous destruction of tissue, and the so-called "collagen" group of diseases including rheumatic fever, disseminated lupus erythematosus and rheumatoid arthritis have been shown to result in elevated serum mucoprotein or serum hexosamine levels (Seibert *et al.*, 1947; Shetlar *et al.*, 1950a; Shetlar *et al.*, 1950b; Lockey *et al.*, 1956; Cohen and Byham, 1950; Galletti *et al.*, 1955; Rosenberg and Schloss, 1949; Shetlar *et al.*, 1952; Boas and Sofer, 1951; Bonomo *et al.*, 1956).

It is possible that the elevations reported in all cases of acute nephritis may well represent a ground substance mucopolysaccharide depolymerization in this condition. However, the possibility that the elevations may be due to impaired renal excretion must be excluded, since it has been shown that in man up to 200 mg. of mucoprotein are normally excreted daily in the urine (Lockey *et al.*, 1956).

Elevation of the serum glycoprotein levels associated with an increased urinary excretion of acid mucopolysaccharides have previously been reported in patients with diabetes mellitus (Jacobs, 1949; Berkman *et al.*, 1953; Gilliland *et al.*, 1954; Nielsen and Poulsen, 1954; Törnblom and Nordström, 1954; Keiding, 1954; Craddock and Kerby, 1955; Andreani and Gray, 1956). Similar elevations are now reported in insulin-controlled diabetics. The failure of insulin acutely to influence the serum glycoprotein levels in this disorder has been recently demonstrated (Rosenlund, 1956), and this finding is confirmed in patients receiving prolonged insulin therapy. It is suggested that both elevations may represent an increased breakdown of connective tissue glycoproteins in diabetes, and that

this abnormality in acid mucopolysaccharide metabolism is brought about by other than an insulin deficiency. The nature of the defect in mucopolysaccharide metabolism in diabetes mellitus is ill understood.

These elevations of the serum mucoprotein and hexosamine levels in approximately one-half of the diabetics correlate well with the alpha globulin increases which have been reported in diabetes mellitus by Keiding (1954) and Ralli *et al.* (1957). Since the alpha globulin fraction has the highest polysaccharide content of the various protein fractions (Seibert *et al.*, 1947) and the mucoprotein moiety has the same electrophoretic mobility as the alpha globulin component (Weimer *et al.*, 1950), it is likely that the elevations noted in this study correspond to the elevations of the alpha globulins obtained by electrophoresis. It is also likely that the alpha 2 globulin increases of "Collagen" disease (Paton *et al.*, 1954) have a similar explanation, and reflect the mucoprotein elevations shown in this study.

Recently, elevations of both mucoprotein and hexosamine levels have been noted in human atherosclerosis (Antonini and Salvini, 1957; Schwartz and Gilmore, 1958), and an elevation of the hexosamine level has been demonstrated in hypercholesterolaemic, atherosclerotic rabbits (Schwartz *et al.*, 1958). Such findings have been interpreted as indicating a connective tissue glycoprotein depolymerization in atherogenesis. The findings in this study of elevations of both fractions in diabetes mellitus suggest that the atherogenic potential of this disorder may be partly explained on this basis. Such an hypothesis is further supported by the demonstration of similar abnormalities in hypothyroidism in which condition atherosclerosis is facilitated (Mancini *et al.*, 1951; Boas and Foley, 1954). Of related interest is the recent observation of Schwartz and Casley-Smith (1958) that in an ethnic group with a low incidence of atherosclerosis, namely, the Australian aborigine, there is an associated low serum mucoprotein level. The observation of an elevated mucoprotein and hexosamine level in one case of nephrosis is of interest in view of the severity of atherosclerosis noted in this disorder.

SUMMARY.

Testicular hyaluronidase has been shown *in vivo* to produce significant elevations of both the serum mucoprotein and hexosamine levels in the rat.

It is suggested on the basis of the specificity of this enzyme that these elevations may reflect an increased connective tissue ground substance depolymerization.

Elevations of both fractions have been noted in one-half of the insulin-treated male diabetics, acute nephritics, the nephrotic syndrome, and in one case each of scleroderma and dermatomyositis.

These elevations, indicative of abnormal mucopolysaccharide metabolism, are interpreted as probably reflecting the degree of depolymerization of ground substance glycoproteins in these disorders.

These abnormalities in some of the insulin controlled diabeties are discussed, and a metabolic defect in diabetes mellitus due to other than an insulin deficiency is suggested.

It is suggested that the facilitation of atherogenesis by diabetes may be explained on the basis of abnormal connective tissue mucopolysaccharide metabolism in this disorder.

REFERENCES.

Andreani, D. V., and Gray, C. H. (1956): Clin. Chim. Acta., 1, p. 7.
Antonini, F. M., and Salvini, L. (1957): Bull. Schweiz. Akad. med. Wiss., 13, p. 216.
Berkman, J., Rifkin, H., and Ross, G. (1953): J. clin. Invest., 32, p. 415.
Boas, N. F., and Foley, J. B. (1954): Proc. Soc. exp. Biol. Med., 86, p. 89.
Boas, N. F., and Sofer, L. J. (1951): J. clin. Endocrinol., 11, p. 39.
Bonomo, E., Sala, G., Amira, A., and Bolobio, C. B. (1956): Acta. med. Scand., 154, p. 361.
Cohen, R. J., and Byham, B. J. (1950): J. lab. clin. Med., 35, p. 869.
Craddock, J. G., and Kerby, G. P. (1955): *Ibid.*, 46, p 193.
Galletti, F., Gelli, G., and Loli-Piccolomini, M. (1955): Acta. tuberc. Scand., 31, p. 185.
Gersh, I., and Catchpole, H. R. (1949): Amer. J. Anat., 85, p. 457.
Gilliland, I. C., Hanno, M. G., and Strudwick, J. I. (1954): Biochem. J., 32, p. 56.
Jacobs, H. R. (1949): J. lab. clin. Med., 34, p. 116.
Johnson, A. R., O'Halloran, M. W., and Hewgill, F. R. (1958): J. Amer. Oil Chem. Soc., in press.
Keiding, N. R. (1954): Proc. Soc. exp. Biol., 86, p. 390.
Lockey, E., Anderson, A. J., and Maclagan, N. F. (1956): Brit. J. Cancer, 10, p. 209.
Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951): J. biol. Chem., 193, p. 265.
Mancini, R. E., Garberi, J. C., and de la Balze, F. A. (1951): Rev. Soc. Argent Biol., 27, p. 285.
Meyer, K. (1954): Connective Tissues in Health and Disease. G. Asboe-Hansen (ed.) E. Munksgaard, Copenhagen.
Nielsen, G. H., and Poulsen, J. E. (1954): Danish med. Bull., 1, p. 70.
Paton, J. B., Robertson, G. K., and Wellby, M. L. (1954): Med. J. Austral., Jan. 23, p. 108.
Ralli, E. P., Barbosa, X., Beck, E. M., and Laken, B. (1957): Metabolism, 6, p. 331.
Rosenberg, C., and Schloss, B. (1949): Amer. Heart J., 38, p. 872.
Rosenlund, B. (1956): Scand. J. clin. lab. Invest., 8, p. 319.
Schwartz, C. J., and Casley-Smith, J. R. (1958): Austral. J. exp. Biol., 36, p. 117.
Schwartz, C. J., and Gilmore, H. R. (1958): Circulation, in press.
Schwartz, C. J., Peters, J. A., and Day, A. J. (1958): Austral. J. exp. Biol., 36, p. 109.
Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W. (1947): J. clin. Invest, 26, p. 90.
Shetlar, M. R., Erwin, C. P., Everett, M. R. (1950a): Cancer Res., 10, p. 445.
Shetlar, M. R., Shetlar, C. L., Richmond, V., and Everett, M. R. (1950b): *Ibid.*, 10, p. 681.
Shetlar, M. R., Schmidt, J. L., Lincoln, R. B., De Vore, J. K., Bullock, J. A., and Hellbaum, A. A. (1952): J. lab. clin. Med., 39, p. 372.
Törnblom, N., and Nordström, K. (1954): Acta. Endocrinol., 17, p. 426.
Weimer, H. E., Mehl, J. W., and Winzler, R. J. (1950): J. biol. Chem., 185, p. 561.
West, R., and Clarke, D. H. (1938): J. clin. Invest., 17, p. 173.
Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M. (1948): *Ibid.*, 27, p. 609.

THROMBIN AND THE THROMBOPLASTIC ACTIVITY OF PLASMA

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Normal human serum is free of antihaemophilic activity. Alexander, Goldstein, Rich, Le Bolloc'h, Diamond and Borges (1954) found this to be due to destruction of the antihaemophilic factor by thrombin. According to Penick, Wagner, Roberts and Brinkhous (1955) this occurs both *in vitro* and *in vivo*.

An investigation of the behaviour of the clotting factors in the presence of thrombin is of interest because, during coagulation of normal blood, amounts of thrombin in excess of that required for fibrin formation are produced.

The following experiments are concerned with the effect of thrombin preparations on the thromboplastic activity of plasma under certain experimental conditions.

Materials.

Fresh human oxalated plasma, poor in platelets, was prepared from venous blood mixed with 1/10 of its volume of 0·1M sodium oxalate, and was stored under sterile conditions, in test tubes plugged with cotton wool, for at least 14 days at room temperature.

A commercial thrombin preparation of bovine origin (Parke-Davis Thrombin Topical) was used and dissolved in 0·145M sodium chloride, and calcium removed with sodium oxalate. Human thrombin preparations were made according to Mellanby (1909) with the following modifications: 50 ml. oxalated human plasma were diluted with 500 ml. ice-cold distilled water, a drop of secondary octanol was added to prevent foaming, and carbon dioxide was bubbled through the ice-cold mixture for 5 minutes. The precipitate was collected by centrifugation and, after drainage, dissolved in 25 ml. of a solution of 0·145M sodium chloride and 0·003M sodium bicarbonate. The pH was kept between 7·2-7·4. The solution was then warmed to 37° C. and 2·5 ml. 0·025M calcium chloride added. Fibrin was removed by winding on a glass rod. Calcium ions were then precipitated by the addition of 0·6 ml. of 0·01M sodium oxalate and the calcium oxalate was removed. This thrombin preparation was stored at — 15° C. It usually contained 180-200 pyrocatechol thrombin units per ml. (Fantl, 1954) which corresponds to 120-135 I.U. thrombin.

A phospholipid (P-lipid) preparation similar to that of Eagle (1934) was made from part of a human brain freed of the meninges, washed free from blood with 0·145M sodium chloride, and ground with acetone until the Liebermann-Burchard cholesterol reaction in the acetone extract remained constant. The acetone was removed by evaporation from the brain powder at 37° C. The residue was extracted with peroxide-free ether and frequently shaken in a stoppered vessel for at least 2 hours. The suspension was filtered and the clear filtrate was evaporated under reduced pressure. The residue was suspended with the aid

[1] The expenses of this investigation were defrayed by a grant from the National Health and Medical Research Council.

of glass beads in 0·145M sodium chloride to produce a 3 p.c. P-lipid concentration. The preparation was distributed in 1 ml. lots and kept stoppered at — 15° C. Activity was found undiminished for several months. Prior to use the stored preparation was diluted 100 times with 0·145M sodium chloride. The calcium chloride solution used was 0·025M and contained 0·086M boric acid.

In this paper the following terms are used with the meanings indicated: *α-haemophilia*: haemorrhagic tendency due to deficiency of α-prothromboplastin (antihaemophilic factor, antihaemophilic globulin, platelet co-factor 1). *β-haemophilia*: haemorrhagic tendency due to deficiency of β-prothromboplastin (P.T.C., Christmas factor). *Pro-accelerin*: prothrombin accelerator, factor V, labile factor, plasma ac-globulin. *Accelerin*: factor VI, serum ac-globulin.

Except that barium sulphate treated plasma (Ba-plasma) was used as source of anti-haemophilic factor, the thromboplastin generation tests were carried out essentially according to Biggs and Douglas (1953).

Determinations of proaccelerin and accelerin activities were carried out on mixtures of Ba-plasma and stored human oxalated plasma after adjustment of the pH to 7·4 with carbon dioxide; otherwise they were made according to the procedure of Quick and Stefanini (1948).

RESULTS.

Five ml. of fresh normal human oxalated plasma were treated with 10 I.U. of human or bovine thrombin per ml. of plasma at room temperature (approximately 20° C.) for 30 minutes. The thrombin-treated oxalated plasmas were compared with untreated plasma for their ability to convert prothrombin. The results are given in Fig. 1.

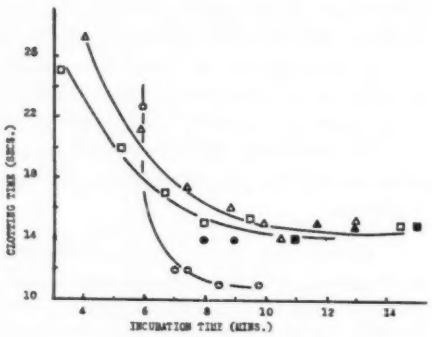

Fig. 1.

(a) Thrombin formation in normal plasma: 0·1 ml. fresh normal human oxalated plasma, 0·6 ml. P-lipid suspension containing 0·18 mg. P-lipid, 0·6 ml. calcium chloride, 0·145M sodium chloride to a total volume of 2·4 ml. Incubated at 28° C. pH 6·5-7·1. Substrate: normal plasma and calcium ○ —, Ba-plasma and calcium ● —.

(b) 0·12 ml. fresh normal human oxalated plasma treated with human thrombin, 10 I.U. per ml. plasma, other additions as under (a). Substrate: normal plasma and calcium △ —, Ba-plasma and calcium ▲ —.

(c) 0·12 ml. fresh normal human oxalated plasma treated with bovine thrombin 10 I.U. per ml. plasma, other additions as under (a). Substrate: normal plasma and calcium □ —, Ba-plasma and calcium ■ —. In each case 0·2 ml. reaction mixture was tested either with 0·2 ml. normal plasma and 0·2 ml. calcium chloride or with 0·2 ml. Ba-plasma and 0·2 ml. calcium chloride.

The results in Fig. 1 show that appreciable thromboplastic activity is present in the thrombin-treated plasma. The added thrombin was absent after the incubation as clotting times longer than 60 seconds were obtained in tests without the addition of P-lipid. In other experiments up to 25 I.U. thrombin per ml. plasma were added, and incubation was prolonged to two hours. The results were not significantly different from those given in Fig. 1. A 20 p.c. dilution of normal

oxalated plasma developed a similar thromboplastic activity as the thrombin-treated plasma.

The antihaemophilic activities of the original and the thrombin-treated plasma were determined after barium sulphate adsorption in a thromboplastin generation test. It has to be added that, for efficient thromboplastin formation, adequate concentration of pro-accelerin is also required (Biggs, Douglas and Macfarlane, 1953). Thrombin converts pro-accelerin into accelerin (Ware, Murphy and Seegers, 1947). Barium sulphate adsorbs only a small fraction of pro-accelerin and antihaemophilic factor but a larger one of accelerin (Ware and Lewis, 1953). Plasmas were treated—

(a) first with thrombin and then adsorbed with barium sulphate; and

(b) First with barium sulphate and then with thrombin and the ability to produce thromboplastic activity was determined. Results are given in Table 1.

TABLE 1.

*Thromboplastin formation in thrombin treated human oxalated plasma**.

Plasma used	Incubation time (min.)	Clotting time (sec.)	Accelerator activity %
Original Ba-plasma	4-6	8-11	100
(a) plasma + thrombin, adsorbed with barium sulphate	<7	40	10-60
(b) Ba-plasma + thrombin	<7	50	140-45

* The thromboplastin generation test was used.

The results given in Table 1 show that the Ba-plasma a and b formed practically no thromboplastin. The accelerin activity of the thrombin-treated plasmas varied considerably; it was higher in Case b at the start, but diminished during the next two hours.

The results given in Table 1 and Fig. 1 indicate that despite the fact that thrombin destroyed more than 98 p.c. of antihaemophilic activity of normal human oxalated plasma it produced thromboplastic activity corresponding to approximately 20 p.c. of normal plasma. It was therefore of interest to compare the activities of normal plasma treated with thrombin and plasma from α-haemophilics with varying degrees of deficiency of α-prothromboplastin. Results are shown in Fig. 2.

It is apparent that the α-haemophilic, whose plasma had 15 ± 5 p.c. antihaemophilic factor, produced less thromboplastic activity than was obtained with normal plasma treated with thrombin which had less than 2 p.c. antihaemophilic activity.

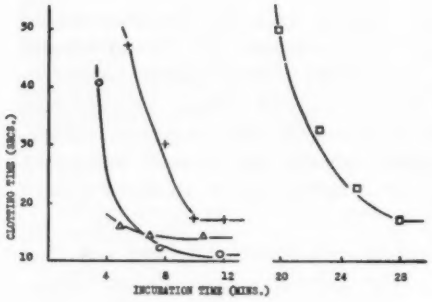

Fig. 2.

Thrombin formation in normal and α-haemophilic plasma:
(a) 0·1 ml. fresh normal human oxalated plasma ○ —.
(b) 0·1 ml. fresh normal human oxalated plasma treated with human thrombin 25 I.U. per ml. plasma for 1½ hours △ —.
(c) 0·1 ml. α-haemophilic plasma (15 ± 5 p.c. α-prothromboplastin) + —.
(d) 0·1 ml. α-haemophilic plasma (< 1 p.c. α-prothromboplastin) □ —.
Substrate: normal human oxalated plasma and calcium. All other experimental conditions similar to those in Fig. 1.

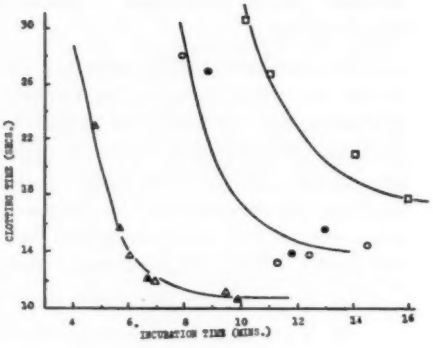

Fig. 3.

Thrombin formation in normal and α-haemophilic plasma.
(a) 0·12 ml. fresh normal human oxalated plasma treated with human thrombin 25 I.U. per ml. plasma for 1½ hours. Substrate: normal plasma and calcium △ —, Ba-plasma and calcium ▲ —.
(b) 0·12 ml. of α-haemophilic plasma (10 p.c. α-prothromboplastin). Substrate: normal plasma and calcium ○ —, Ba-plasma and calcium ● —.
(c) 0·12 ml. α-haemophilic plasma (b) treated with human thrombin 25 I.U. per ml. plasma for 1½ hours. Substrate: normal plasma and calcium □ —. All other experimental conditions similar to those in Fig. 1.

The effect of thrombin treatment on the oxalated plasma of an α-haemophilic whose plasma had 10 p.c. α-prothromboplastin is recorded in Fig. 3.

It is seen that the normal plasma pre-treated with thrombin gave shorter clotting times than the α-haemophilic plasma and that thrombin treatment of the latter produced some reduction in activity.

The experiments suggest that thrombin reacted with a component of normal plasma, most likely the antihaemophilic factor. Fig. 3 shows that the effect on clotting is the opposite to that expected by a destructive process. In fact, the experiments indicate the production of a thromboplastin-like material.

In order to show whether only plasma rich in antihaemophilic activity is measurably influenced by thrombin, experiments were carried out with the plasma of a β-haemophilic who had a complete deficiency of β-prothromboplastin but a normal α-prothromboplastin activity (Fig. 4).

The results in Fig. 4 indicate that the rate of formation and the amount of thrombin produced in the β-haemophilic plasma was low and not significantly altered by treatment with thrombin. Experiments in Fig. 3 and Fig. 4 indicate

that thrombin did not introduce nor produce a thromboplastic material active in the conversion of prothrombin, and show that the thrombin activated material is not a complete thromboplastin, otherwise shorter clotting times would have been found in the thrombin-treated, prothromboplastin-deficient plasmas.

For the assay of the antihaemophilic factor plasma or plasma fractions free of it, but with adequate proaccelerin activity are required.

It seemed therefore of interest to determine whether Ba-plasma treated with thrombin could be substituted for plasma completely deficient in the antihaemophilic factor. Normal Ba-plasma as source of the antihaemophilic factor was added to the Ba-plasma treated with thrombin and to Ba-plasma of an α-haemophilic with a complete deficiency (Fig. 5). Results given in Fig. 5 were obtained.

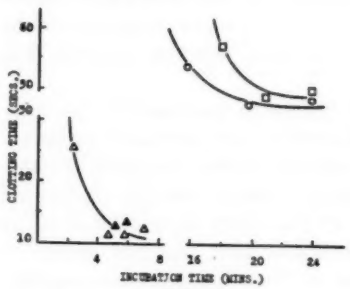

Fig. 4.

Thrombin formation in normal and β-haemophilic plasma.

(a) 0·12 ml. fresh normal human oxalated plasma treated with human thrombin 25 I.U. per ml. plasma for 1 hour. Substrate: normal human oxalated plasma and calcium △—, Ba-plasma and calcium ▲—.

(b) 0·12 ml. β-haemophilic oxalated plasma (< 1 p.c. β-prothromboboplastin). Substrate: normal plasma and calcium ○—.

(c) 0·12 ml. β-haemophilic plasma (b) treated with human thrombin 25 I.U. per ml. plasma for 1 hour. Substrate: normal plasma and calcium □—. All other experimental conditions similar to those in Fig. 1.

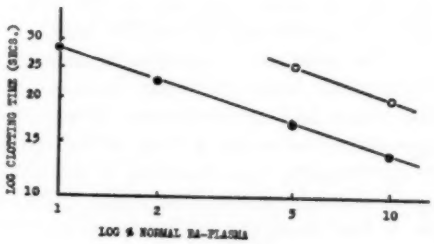

Fig. 5.

Thromboplastin formation in plasma mixtures:

(a) 0·12 ml. Ba-plasma of an α-haemophilic (< 1 p.c. α-prothromboplastin) 0·6 ml. P-lipid suspension containing 0·18 mg. P-lipid, 0·06 ml. normal human serum. Addition of normal Ba-plasma diluted with 0·145M sodium chloride containing 0·001M sodium oxalate, 0·6 ml. calcium chloride and 0·145M sodium chloride to total volume of 2·4 ml. ●—.

(b) 0·12 ml. fresh normal human Ba-plasma treated with 25 I.U. thrombin per ml. for 1½ hours. Other additions similar to (a). The shortest clotting times are given ○—.

The results of thromboplastin generation tests given in Fig. 5 show that a similar addition of normal Ba-plasma to the α-haemophilic Ba-plasma gave shorter clotting times than with normal Ba-plasma treated with thrombin.

The proaccelerin activity of the α-haemophilic plasma was 100 p.c., the accelerin activity of Ba-plasma treated with thrombin dropped from 140-45 p.c.

during the experiment. In other experiments it was established that a concentration of 40 p.c. proaccelerin gave clotting times only slightly shorter than with 100 p.c. From the results given in Fig. 5 it is apparent that the accelerin activity of thrombin-treated plasma is inadequate for maximum thromboplastin formation.

DISCUSSION.

Several workers have been interested in the variation of antihaemophilic activity of plasma and the activity of various isolated fractions under a number of experimental conditions.

Johnson and Seegers (1954) observed loss of activity of "platelet co-factor 1" in the presence of thrombin and calcium ions, but none if calcium was absent. Douglas (1956) found a normal rate of disappearance of antihaemophilic factor in the absence of platelets. However, Penick (1957) noticed progressive loss of antihaemophilic activity in the absence of calcium, but the reaction was markedly accelerated by calcium ions.

The presented experiments indicate that thrombin inactivated the antihaemophilic factor of normal human oxalated plasma and produced a derivative which had approximately 20 p.c. of the activity of the untreated plasma. The derivative is not a fully active thromboplastin because for the conversion of prothrombin into thrombin it still requires all the other factors involved in blood thromboplastin formation. This thrombin-activated material seems to be derived from the antihaemophilic factor but no longer has antihaemophilic properties since the activity has been lost after adsorption with barium sulphate.

Development of thromboplastic activity during storage of citrated blood, despite the loss of antihaemophilic activity, has been observed by Soulier and Wartelle (1956).

Proaccelerin is required for efficient formation of blood thromboplastin, yet plasma which contained accelerin and was free of antihaemophilic activity could not be used as substitute for a-haemophilic plasma, because the addition of normal Ba-plasma as source of the antihaemophilic factor produced longer clotting times in the accelerin-containing plasma than in the a-haemophilic plasma. This seems remarkable because accelerin activity as determined in the presence of brain thromboplastin during prothrombin conversion shows higher activity than proaccelerin.

It should be pointed out that although the thrombin preparations clotted plasma in 5-6 secs. they were very impure. Nevertheless, there is no reason to assume that the described observations are due to any other component of the preparations than to thrombin.

SUMMARY.

Thrombin inactivates the antihaemophilic activity of normal human oxalated plasma and produces a thromboplastic-like substance which converts prothrom-

bin into thrombin, if the other clotting factors essential for blood thromboplastin formation are present.

The accelerin activity produced by thrombin from normal human oxalated plasma is inadequate for efficient blood thromboplastin formation.

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REFERENCES.

Alexander, B., Goldstein, R., Rich, L., Le Bolloc'h, Anne G., Diamond, L. K., and Borges, W. (1954): Blood, 9, p. 843.
Biggs, R., and Douglas, A. S. (1953): J. clin. Path., 6, p. 23.
Biggs, R., Douglas, A. S., and Macfarlane, R. G. (1953): J. Physiol., 122, p. 538.
Douglas, A. S. (1956): Blood, 11, p. 423.
Eagle, H. (1934): J. gen. Physiol., 18, p. 531.
Fantl, P. (1954): Biochem. J., 57, p. 416.
Johnson, S. A., and Seegers, W. H. (1954): Revue Haematologique, 9, p. 529.
Mellanby, J. (1909): J. Physiol., 38, p. 28.
Penick, G. D., Wagner, R. N., Roberts, H. P., and Brinkhous, K. M. (1955): Fed. Proc., 14, p. 415.
Penick, G. D. (1957): Proc. Soc. exp. Biol. Med., 96, p. 277.
Quick, A. J., and Stefanini, M. (1948): J. lab. clin. Med., 33, p. 819.
Soulier, J. P., and Wartelle, O. (1956): Vox Sanguinis, 1, p. 110.
Ware, A. G., Murphy, R. C., and Seegers, W. H. (1947): Science, 106, p. 618.
Ware, A. G., and Lewis, M. L. (1953): Fed. Proc., 12, p. 286.

THE EFFECTS OF HEAVY METAL IONS ON THE HUMAN ERYTHROCYTE

II. THE EFFECTS OF LEAD AND MERCURY

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Previous work has shown that many metal ions radically interfere with the ionic stability of the human red cell and lead to a prolytic K^+ efflux from the cell (Vincent and Blackburn, 1958). Of the several metals investigated, salts of lead were found to affect the cell in a unique way. They caused a rapid diminution in cell volume, and a concomitant K^+ loss from the cell at first unbalanced by a Na^+ influx. Later, Na^+ ions entered the cell and a prolytic swelling resulted. Mercuric salts, on the other hand, disturbed the ionic metabolism in that the Na^+ influx was much greater, and the shrinkage less, than in the case of lead. With these differences in mind, further investigations comparing the action of lead and mercury have been carried out.

The present studies concern the effects of the duration of action of lead and mercury on the loss of K^+ from the cell, the uptake of each metal by the cell, and the changes in cellular volume. In addition, the microscopic appearances of erythrocytes after exposure to lead are described.

Methods.

The methods for the separation and suspension of the red cells, and for the estimation of red cell volume, supernatant K^+ and haemoglobin, were identical with those described previously (Vincent and Blackburn, 1958). All glassware used was freed of Pb and Hg contamination by two washings with 50 p.c. redistilled nitric acid, followed by two rinsings with triple glass-distilled water.

To study the effects of time of metal action, mixtures of red cells and isotonic solution of sodium acetate (for Pb) or sodium chloride (for Hg) each with 0·025 M glucose, were prepared. At zero time, isotonic solutions of lead acetate or mercuric chloride were added, in the ratios of 0·2 µmoles and 0·8 µmoles per ml. packed cells respectively. In the suspension so prepared, the ratio by volume of cells to suspending fluid was the same as in previous studies, namely 1 to 5. Aliquot samples were withdrawn at given times and after centrifugation the supernatant was assayed for K^+ and Pb or Hg.

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Changes in cell volume were estimated by a similar technique except that packed cells formed one-half of the final suspension. Haematocrit estimations were limited to 5 minutes centrifugation at 3,000 r.p.m., because samples were withdrawn at 10-minute intervals. These conditions are far from adequate for packing of the cells to a constant volume, but centrifugation at uniform speed for the same time ensured that readings for each sample were comparable.

Estimations of the Erythrocyte Sedimentation Rate (ESR), of red cells treated with lead or mercury, were made. To 1·5 ml. of whole de-calcified blood, 0·05 ml. of an isotonic Hg solution or 0·4 ml. of an isotonic Pb solution, was added, and controls were prepared by the use of 0·05 or 0·4 ml. of 0·85 p.c. NaCl in place of the metal solution. The concentration of the Hg solution was 10 μmoles/ml., and of the Pb solution 0·25 μmoles/ml. so that the final concentrations were respectively 0·75 and 0·15 μmoles/ml. packed cells. After incubation for forty minutes at 37° C., the ESR was determined in Westergren tubes at room temperature.

The uptake of heavy metal from the solution was determined by estimation of the concentration remaining in the supernatant at increasing times after exposure to the cells. Pb was estimated by the mixed colour Dithizone method, described by Sandell (1950). Hg was estimated by a method based on the Cholak and Hubbard digestion procedure and the Laug and Nelson complexing technique (Barrett, 1955, 1956). Recovery of added Hg was of the order of 98 to 102 p.c., with an accuracy of 1 p.c.

Microscopic studies of Pb-poisoned cells were carried out:

(i) In ordinary and plane-polarized light, using a Zeiss microscope fitted with crossed Nicol prisms;

(ii) Under fixed phase constrast, using a Zeiss "Lumipan" microscope;

(iii) Under variable phase contrast, using a Wild "Varicolor" instrument.

The cells were freshly collected and washed as previously described, and re-suspended in an equal volume of their own plasma. Lead acetate was added either (a) in the ratio of 0·2 μmoles/ml. packed cells, before examination on the slide, or (b) as a drop of 0·15M lead-agar, set on the slide to which the cells were added. The former method was designed to study the change in appearance of the cells under conditions comparable with those used in other studies; but the earliest shape changes were missed. The latter was developed so that the Pb concentration in the suspending fluid increased slowly, as Pb diffused into solution from the agar, allowing observation of the early microscopic changes. Spontaneous crenation and sphering was checked by the use of a control slide and prevented in large degree by the suspension of the cells in their own plasma on paraffined slides and cover-slips.

EXPERIMENTAL.

The loss of potassium subsequent to the exposure of red cells to lead or mercury.

The technique for the study of the rate of loss of K^+ from the red cell after its exposure to either Pb or Hg has been described above. This method of aliquot sampling from a stock reacting mixture was found to be a most satisfactory one, allowing of reproducibility of results on many occasions. The use of single tube methods was found to be beset with variations in results which were unavoidable and independent of time.

Lead. The loss of K^+ from red cells at increasing times after exposure to 0·2 μmoles of Pb per ml. packed cells is set out in Table 1, and Fig. 1 has been

fitted to the points by inspection as the apparent line of best fit. Statistical analysis reveals that the points as a whole depart very significantly from a straight line or a quadratic, but that two straight lines fit the points without a significant residual variance. Two lines fitted between 0 and 30 minutes, and 40 and 110 minutes respectively, represent the closest statistical approximation to the experimental points. The former has a slope of 1·65 and the latter approximates very closely to the horizontal with a slope of 0·07.

In the period between 36 and 40 minutes, extrapolations of the two calculated lines depart from the experimental points and it is to be expected that a smooth continuous curve as shown in Fig. 1 represents all the points most

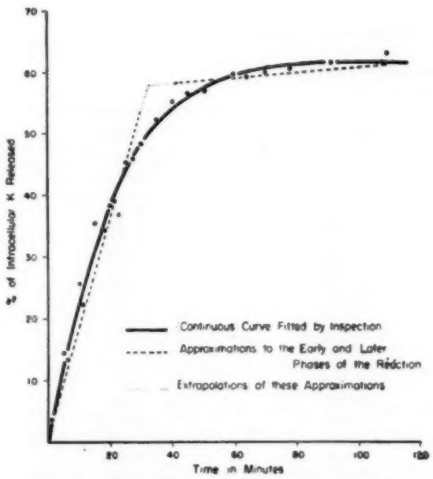
% of Intracellular K Released
Time in Minutes
Continuous Curve Fitted by Inspection
Approximations to the Early and Later Phases of the Reaction
Extrapolations of these Approximations

Fig. 1. The release of K^+ from the red cell compared with the duration after exposure to Pb. Human red cells were exposed to 0·2 μmoles Pb/ml. packed cells at 37° C. and serial estimations of the K^+ released into the supernatant were carried out.
Ordinate: K^+ released expressed as a percentage of the initial intracellular K^+ concentration.
Abscissa: Time in minutes after exposure of the cells to Pb.

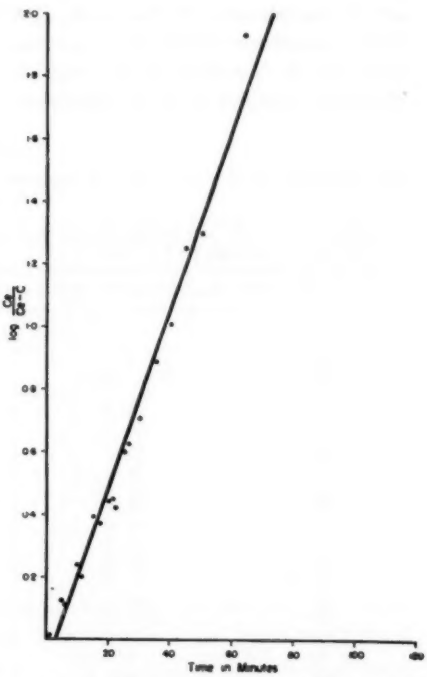

Fig. 2. The approximation of the loss of K^+ from the Pb-poisoned red cell to a first order reversible reaction. Experimental conditions as in Fig. 1.
C: K^+ released from the red cell during t minutes after exposure to Pb.
Ce: K^+ released from the red cell by equilibrium.

closely. The two lines, however, serve the purpose of demonstrating the two phases of the relationship as mathematical approximations. The early phase is apparently one of rapid K^+ loss, as indicated by the steep slope of the line, whereas the approximation of the slope of the later phase to zero suggests that a state of virtual equilibrium has been achieved.

The appearance of the curve prompted a comparison of the data with the findings expected in a first order reversible reaction. The formula for such a reaction may be expressed

$$(k_1 + k_2)\, t = 2{\cdot}303 \log \frac{Co - Ce}{C - Ce} \quad \text{(i)}$$

where k_1 and k_2 are the velocity constants for the forward and reverse reactions, Co, C and Ce are the concentrations of the reactant at 0 minutes, t minutes and at equilibrium (Höber *et al.*, 1945). In this case the values for Co, C and Ce are best derived from the supernatant $[K^+]$, rather than from the intracellular $[K^+]$ which is influenced by changes in cell volume. If the concentration is expressed throughout as a percentage of the initial intracellular $[K^+]$, Co can

TABLE 1.

Data comparing the loss of K from red cells with the time after exposure to 0·2 μmoles Pb/ml. packed cells at 37°C.

All values for K^+ are expressed as percentages of the initial intracellular K^+.
C is the percentage lost at time t.
Ce is the percentage lost at equilibrium, approximately 60 p.c.
Co is the initial intracellular K^+ concentration, i.e. 100 p.c.

Time (mins.)	C mean values	$\frac{Ce}{Ce-C}$	$\log \frac{Ce}{Ce-C}$
1	3·6	1·07	0·03
5	14·2	1·31	0·12
6·5	13·2	1·28	0·11
10	25·8	1·75	0·24
11	22·1	1·59	0·20
15	35·4	2·44	0·39
18	34·2	2·32	0·37
20	38·1	2·74	0·44
21	38·9	2·84	0·45
22·5	37·0	2·60	0·42
25	45·1	4·02	0·60
27	46·0	4·30	0·63
30	48·4	5·16	0·71
35	52·2	7·70	0·89
40	55·2	12·5	1·08
45	56·6	17·7	1·25
51	57·0	20·0	1·30
53	63·4	—	—
60	59·9	600	2·78
64	59·3	86	1·93
70	60·0		
78	60·6		—
91	61·3	—	—
109	63·4	—	—

be taken as 0 and Ce as 60. Since C is always less than Ce, the equation (i) can be rewritten:

$$(k_a + k_b)\, t = 2{\cdot}303 \log Ce/Ce - C \quad \ldots\ldots \quad \ldots\ldots \quad \ldots\ldots \quad (ii)$$

k_a and k_b being the velocity constants for the K^+ influx and efflux respectively.

Calculations of the term $Ce/Ce - C$ are set out in Table 1, and the linear relationship of its log with time is shown in Fig. 2. The sum $k_a + k_b$ is 0·063 and since the ratio $k_a/k_b = 2{:}3$, k_a is 0·024 and k_b is 0·036.

Mercury. The results of similar studies using 0·8 μmoles Hg/ml. packed cells are set out in Table 2 and Figs. 3 and 4. A smooth continuous curve has been fitted by inspection, and as was the case with Pb it is to be expected that this is the closest representation of the experimental points. Again, statistical analysis shows that a straight line or a quadratic fails to fit the points without a significant residual variance, and that two straight lines represent a satisfactory approximation to the curve. If fitted to points between 0 and 20 and 40 and 60 minutes respectively, they yield the least residual variance.

TABLE 2.

The release of K from red cells compared with the duration of their exposure to 0·8 μmoles Hg/ml. packed cells at 37°C.

All values for K are expressed as percentages of the initial intracellular K concentration, the symbols C, Ce, Co having the same meaning as in Table 1. Ce approximates to 82 p.c.; Co is 100 p.c.

Time (mins.)	C mean values	$\frac{Ce}{Ce-C}$	$\log \frac{Ce}{Ce-C}$
6	32·4	1·72	0·24
11	51·2	2·66	0·42
16	63·4	4·40	0·64
21	68·9	6·25	0·80
26	74·1	10·3	1·01
31	73·6	9·8	0·99
37	75·6	12·8	1·07
42	80·2	45·6	1·66
50	82·3	—	—
63	82·3	—	—

As was the case with Pb, the experimental points depart markedly from the extrapolations of the two lines between 20 and 40 minutes, but outside these times they approximate closely to the curve. The early slope of 3·3 suggests a very rapid nett efflux of K^+ from the red cell, and the later slope of 0·006 is very close to an equilibrium state.

Analysis showed that these data, too, approximated to the findings to be expected for a first order reversible reaction. Comparison of the expression log $Ce/Ce - C$ (q.v.), with time, showed that a linear relationship existed between the two (Fig. 4). The sum of the velocity constants, k_a and k_b was found to be 0·09, k_a being 0·07 and k_b 0·02.

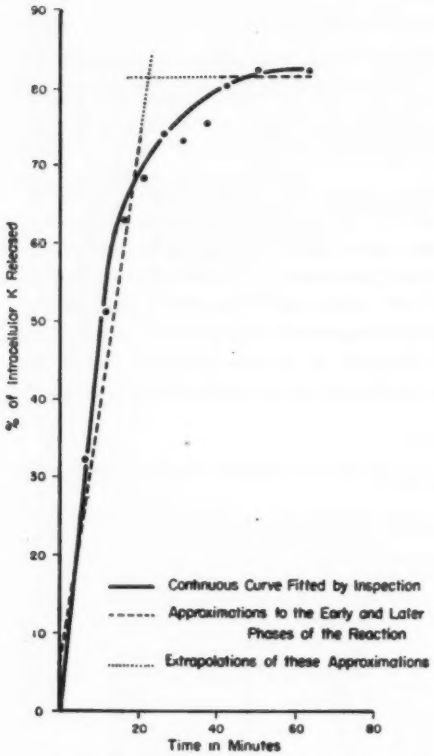
% of Intracellular K Released
Time in Minutes
Continuous Curve Fitted by Inspection
Approximations to the Early and Later Phases of the Reaction
Extrapolations of these Approximations

Fig. 3. The release of K^+ from the red cell compared with the duration after exposure to Hg. Human red cells were exposed to 0·8 μmoles Hg/ml. packed cells at 37° C. and serial estimations of the K^+ released into the supernatant were carried out.
Ordinate: K^+ released, per cent. initial intracellular K^+ concentration.
Abscissa: Time in minutes after exposure of cells to Hg.

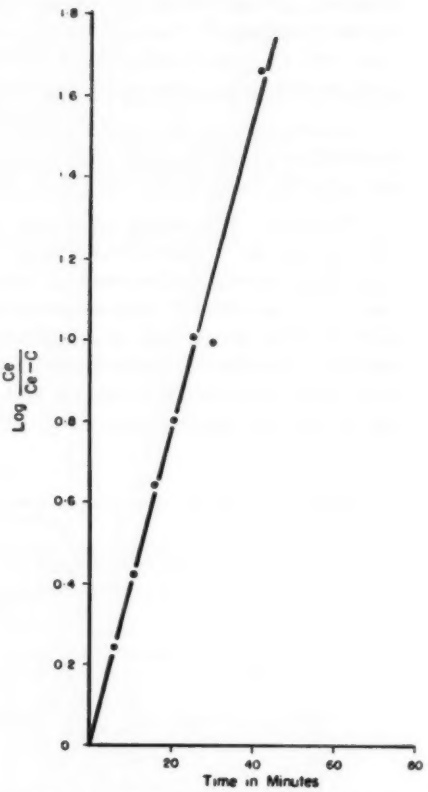

Fig. 4. The approximation of the loss of K^+ from the Hg-poisoned red cell to a first order reversible reaction. Experimental conditions as in Fig. 3.
C: K^+ released from the red cell during t minutes after exposure to Hg.
Ce: K^+ released from the red cell by equilibrium.

The uptake of lead or mercury by the red cell.

As early as could be ascertained after the exposure of red cells to either Pb or Hg, the metal had disappeared from the supernatant to a significant degree. Controls precluded the possibility that the metal had disappeared in any way other than by association with the red cells. During a long subsequent period of incubation the concentration of either metal in the supernatant was not significantly altered.

Within five minutes of the exposure of red cells to Pb, only 10 p.c. of the Pb added could be detected in the supernatant. The mean of all values for the supernatant Pb during the course of 109 minutes incubation at 37° C. was 10 ± 2 p.c. of the added metal. The standard deviation, equivalent to $\pm 0{\cdot}2$ μg./ml. supernatant was less than the experimental error of the Pb estimation ($\pm 0{\cdot}4$ μg./ml.).

Similarly, all but $9{\cdot}4 \pm 0{\cdot}2$ p.c. of the Hg added to red cells had disappeared from the supernatant within six minutes and this concentration remained unaltered during the subsequent incubation.

The changes in shape and volume of red cells after exposure to lead or mercury.

The much greater depression of haematocrit readings of red cells treated with Pb than of those treated with Hg has been described in the first paper of this series. Here, the changes were followed with relation to the time after exposure of the cells to the metal.

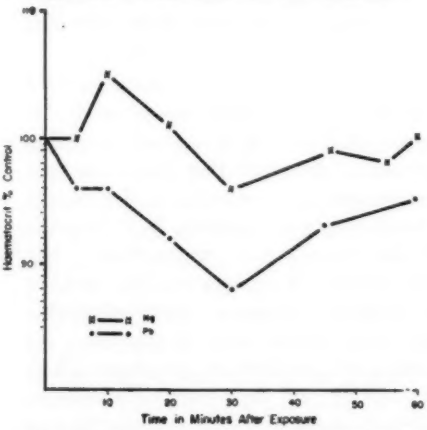

Fig. 5. Comparison of the haematocrit readings at different times after the exposure of red cells to 0·2 μmoles or 0·8 μmoles Hg per ml. packed cells. Temperature, 37° C. All values are expressed as percentage of controls.

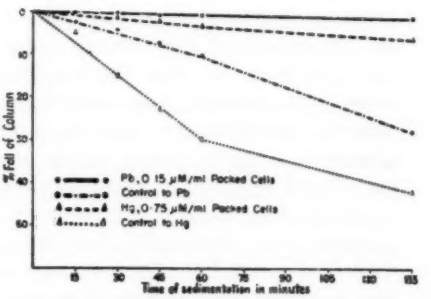

Fig. 6. The effect of Pb and Hg on the ESR of human red cells. Rapidly sedimenting red cells were incubated with 0·15 μmoles Pb, or 0·75 μmoles Hg, per ml. packed cells for 40 minutes at 37° C. Sedimentation over the next 135 minutes was then as shown. Controls to both Pb and Hg were set up; their more rapid sedimentation is shown in the lower two lines.

The results are set out in Fig. 5. Cellular volume decreases rapidly after the addition of Pb and, in terms of five-minute centrifugation, reaches a minimum (90 p.c. of original volume) about 30 minutes later. Thereafter the volume increases. There is also a decrease in cell volume with Hg at about 30 minutes (96 p.c. of original volume), but it returns to normal in 60 minutes.

Fig. 6 compares the ESR of Pb and Hg treated rapidly sedimenting cells with appropriate controls. It will be seen that the sedimentation of treated cells proceeds at a much slower rate than the sedimentation of untreated ones. Both Pb and Hg produce this effect, suggesting that each leads to sphering of the red cells which interferes with rouleaux formation.

Microscopic observations on the appearance of red cells exposed to the action of lead.

Many workers have observed spontaneous conversion of disc-shaped cells to spheres when suspended in saline between plain glass surfaces (Ponder, 1948). This sphering, which is due to the removal of adsorbed serum albumin from the red cell making the latter more susceptible to a rise in pH, can be prevented by suspending the cells in serum, and by coating the glass surfaces with paraffin. In these studies, these measures were adequate to prevent spontaneous sphering of control preparations.

Disc-sphere changes. Spherical forms began to appear immediately following the exposure of red cells to lead. These changes were best observed when discs at the end of rouleaux became swollen and separated as spheres.

With phase contrast illumination, a red cell seen in plan appeared as a dark circle surrounded by a highly refractile ring. Simultaneously with the sphering of cells from rouleaux, the diameter of the cells seen in plan decreased and the width of the refractile ring increased. The fact that these changes were simultaneous, that the resulting appearances were similar and that the subsequent changes were identical for each, strongly suggests that in each case the transformation was the same, namely, from a disc to a sphere. The process was rapid, starting within about 30 seconds of exposure to Pb, and the majority of cells had changed within a few minutes. However, some cells failed to change their shape for very much longer times, up to 10 to 15 minutes. No crenation was observed in any cell preceding its swelling to a sphere. The spheres decreased in size after the addition of Pb but some were observed to start swelling again within 10 minutes. This swelling, which was prolytic, was generalised in the cell population in about 20 minutes. Although the decrease in the volume of Pb-treated cells is very marked (20 p.c.) evidence of bi-refringence, which might have been expected due to a para-crystalline state of the intracellular haemoglobin, was not observed.

Crenation. Two types of apparently crenate cells were observed. The first was a hexagon, derived from, and of the same size as, a spherical cell. A small, highly refractile body could be seen in each of the angles of the hexagon at first, but with the passage of time they disappeared. Some of these bodies were extruded into the medium, and others merely became invisible. This type of cell underwent haemolysis at the same time as, and under the same conditions as, a sphere.

The second type of crenated cell appeared when high concentrations of Pb (0·15M) were added to the red cell system. It was small—roughly half the size of adjacent spherocytes—and highly refractile. Its surface was very irregular, resembling a chestnut. Although these cells were observed for considerable lengths of time, no further change in them could be detected microscopically.

Haemolysis. With high concentrations of Pb, haemolysis was observed in both the spherical and hexagonal crenate forms of cell, but not in the small crenate type. First, the cell lost its refractibility, becoming but a dark shadow, and secondly, this shadow gradually faded from sight, leaving no obvious trace of where the cell had been.

These two phases may correspond respectively to the "fading time" and the "stromatolytic" phases described for haemolytic systems (Ponder, 1948). If the observed refractibility *is* due to the presence of haemoglobin in the cell, its disappearance would correspond to the loss of pigment, characteristic of the "fading period". The "stromatolytic" phase involves the disintegration of the cell ghost, which could correspond to the observed disappearance of the non-refractile cell residue.

DISCUSSION.

Both Pb and Hg cause a considerable prehaemolytic loss of K^+ from the red cell, the nett efflux proceeding rapidly to an equilibrium relationship. The approximation of the data for both metals to first order reversible reactions has been demonstrated, implying that the nett loss of K^+ proceeds as the sum of an efflux proportional to the intracellular $[K^+]$ and an influx proportional to the extracellular $[K^+]$. Joyce *et al.* (1954) have also found that the K^+ loss from rabbit red cells poisoned with Pb, Hg or Au bore a similar relationship to time, although Passow and Tillman (1956) claim that the loss of K^+ from Pb-poisoned red cells is not an exponential relationship. Such a state of affairs might exist if the passage of ions in either direction were governed by simple diffusion.

The differences between intra- and extra-cellular K^+ concentrations observed at equilibrium may then be due to a Donnan phenomenon, but the fact that the ratio between the two depends at least in part on the concentration of the metal added suggests that other factors may be operative. The relatively huge losses of K^+ induced by both Pb and Hg without haemolysis are clearly quite different from those due to lysins such as saponin and digitonin, in which K^+ loss cannot be followed for long before haemolysis supervenes.

The consensus of opinion seems to be in favour of an active mechanism transporting K^+ into the cell, and another transporting Na^+ out, in both cases against a diffusion gradient (Prankerd, 1955). Solomon (1952) has produced evidence that both the influx and efflux of K^+ proceed by the same path (at 37° C.), each requiring energy, although some workers (e.g. Joyce *et al.*, 1954)

believe that only the influx is active, and still others (Maizels, 1949; Flynn and Maizels, 1950; Harris and Maizels, 1951) that K^+ homeostasis is only the passive concomitant of an active Na^+ exclusion.

It is generally agreed that energy is required for the maintenance of cationic homeostasis, Solomon (1952) estimating the requirement in human red cells as 8·8 calories/litre cells/hour for both Na^+ and K^+, compared with a yield of 110 calories from glycolysis by the same volume of cells in the same time. The figure seems to be much higher in nucleated (Duck) red cells (Tosteson and Robertson, 1956). In both these studies, it is suggested that K^+ influx and efflux are coupled in some way, the energy derived from the outflow being at least partly available for the inward transport of the ion. On the other hand, it seems that K^+ movements are thermodynamically independent of Na^+ movements.

Most workers believe that glucose is the substrate utilized in the maintenance of these active fluxes, the inhibitory effect of fluoride ions and iodoacetate (Eckels, 1954; Harris and Maizels, 1951) and the similarity of the activation energy for K^+ transport and glucose metabolism (Solomon, 1952), supporting this view. Greig and Holland (1951) believe that the protection certain choline esters confer against haemolysis points to their metabolism by the red cell being essential in the maintenance of K^+ and Na^+ homeostasis, although Wilde (1955) casts some doubt on the hypothesis.

Heavy metals could interfere with either the active transport mechanisms themselves, or with the generation and transfer of the energy necessary for their function, or with both. Green (1956), having shown that irradiated red cells maintain glycolysis but still lose K^+, suggests that interference with either the membrane or the metabolism of the red cell may be sufficient to disrupt cationic homeostasis. Harris (1957) believes that the rate of loss of K^+ from red cells treated with 30 μM NaF (Eckels, 1954) is analogous to the findings of Joyce *et al.* (1954) for Pb, implying that Pb affects the glycolysis of the cells. We cannot entirely agree with the comparison, however, for we find that the loss of K^+ is rapid in the early stages, in contradistinction to Eckels' finding of an early slow phase. In agreement with Eckels' data, however, we did find that 60-80 p.c. of the total cell K was lost at equilibrium.

The fact that Pb affects the K^+ metabolism of the red cell to a greater extent than the Na^+ metabolism suggests that it is exerting part of its effect directly on K^+ transport mechanism. This finding certainly makes it very unlikely that K^+ homeostasis is nothing but a passive correlate of an active Na^+ transport, as suggested by Maizels (1949). On the other hand, Hg, which disturbs both K^+ and Na^+ homeostasis, may do so by affecting either the transport mechanism for each, or a common energy supply basic to their proper functioning. In this regard, the interesting observation by Joyce *et al.* (1954) of a rapid early influx of ^{42}K into rabbit red cells after exposure to Pb, and, to a lesser extent after Hg,

is possibly significant. This they interpreted as an acceleration of the influx of K^+ in reaction to a loss of K^+ due to increased permeability of the cell wall, the "pump" being unable to continue for long at this rate. The lower ^{42}K influx with Hg, however, reflected a degree of direct inhibition of the "pump" not seen with Pb.

All things being considered, it would seem unlikely that Pb interferes with the generation or transfer of energy to the mechanisms controlling K^+ transport. On the contrary, the evidence suggests that this metal exerts a direct effect on the mechanisms themselves, and the ^{42}K studies discussed above point to an accelerated efflux rather than an inhibited influx. Evidence to be presented in the next paper will show that lipid is required for the association of the metal with the cell. Solomon's hypothetical model for K transport requires the participation of a lipoid soluble, non-lipoid dissociable anion as a carrier, and it may well be that Pb, through a lipid binding, affects this mechanism.

Aub *et al.* (1925), in a study of various species, found that Pb exerted effects on the cell surface – including a decrease in cell volume – only with K^+-rich erythrocytes. With the exception of horse red cells, those which failed to respond in this way were typical K^+-poor red cells. Behrens and Pachur (1927) found that K^+-rich red cells bind more Pb than K^+-poor types, and in the present study the loss of intracellular K^+ has been associated with a binding of Pb with the cell. The sum of this evidence suggests that Pb may be bound to the cell surface at a site concerned in K^+ transport.

It is not possible to say whether Hg acts on the K^+ transport mechanism itself or on energy supplies. If it is accepted that both K and Na transport are energy dependent the effect of Hg on each could be mediated by disturbance of a common energy supply. Hg certainly shows fewer effects on the cell surface than Pb, no changes in osmotic resistance, fragility or surface "stickiness" having been reported, but the sphering reported here and an agglutination of Hg treated red cells (Jandl and Simmons, 1957) indicate some action in this respect. It is pure conjecture whether such lesser surface effects would be compatible with an action of Hg restricted to the cell membrane, or whether one must look to other mechanisms for the K^+ loss and Na^+ gain. The similarity of the pattern of K^+ efflux for the two metals does not necessarily mean that their action is similar; such a pattern would be expected with dysfunction of the active transport mechanism from any cause.

The decrease in cellular volume with Pb has been related to the duration of incubation, a minimum reached at 30 minutes being followed by a return almost to initial size. Hg was found to produce a much slighter effect. Sphering of cells exposed to both metals was indicated by a depression in the ESR of rapidly sedimenting red cells, this change also being observed microscopically in the case of Pb. These latter were very similar to findings reported by Aub *et al.* (1925).

Selwyn and Dacie (1954) found that there was a greater loss of K from red cells of persons with hereditary spherocytosis than from normal erythrocytes, when both were incubated in the absence of glucose. Furthermore, the spherocytes shrank before lysis. Prankerd (1955) suggested that both the K^+ loss and the spherical shape may be due to deficient carbohydrate and phosphorus metabolism – the latter especially affecting the stroma. Pb, and possibly Hg, may interfere at the cell surface with a mechanism basic to the maintenance of cell shape and K content.

The possibility that the two stages observed microscopically in the lysis of the cell may correspond to the "fading time" and the "stromatolytic phase" described by Ponder (1948) has been considered. The small crenate cell described above may represent one in which the cell membrane was so completely disorganised by the metal that further change was impossible. Ponder and Barreto (1957) suggest that differences in the appearance of red cell ghosts are dependent on the structure of the parent red cell, and this may well be the explanation in this case. The finding of this cell supports the contention that red cells react to Pb as an heterogeneous population (Passow and Tillman, 1956).

SUMMARY.

The relationship between the loss of K^+ from the red cell and the time after its exposure to Pb and Hg has been described.

Both Pb and Hg are bound to the red cell to the extent of *ca* 90 p.c. within 5 minutes of exposure.

Sphering of Pb and Hg-treated red cells has been demonstrated by a decrease in the ESR.

The decrease in cell volume after addition of Pb has been followed with time and compared with Hg.

The microscopic appearance of Pb poisoned red cells has been described.

Some implications of these results, especially with respect to active ion transport, are discussed.

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REFERENCES.

Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P. (1925): Medicine, 4, p. 1.
Barrett, F. R. (1955): Med. J. Austral., 2, p. 411.
Barrett, F. R. (1956): Analyst, 81, p. 294.
Behrens, B., and Pachur, R. (1927): Arch. f. exper. Path., 122, p. 319.
Eckels, R. E. (1954): Amer. J. Physiol., 179, p. 632.
Flynn, F., and Maizels, M. (1950): J. gen. Physiol., 110, p. 301.
Green, J. W. (1956): J. cell. comp. Physiol., 47, p. 125.
Greig, M. E., and Holland, W. C. (1951): Amer. J. Physiol., 164, p. 423.
Harris, E. J., and Maizels, M. (1951): J. gen. Physiol., 113, p. 506.
Harris, E. J. (1957): Ann. Rev. Physiol., 19, p. 13.
Höber, R., Hitchock, D. I., Bateman, J. B., Goddard, D. R., and Fenn, W. O. (1945): Physical Chemistry of Cells and Tissues. Blakiston, New York.
Jandl, J. H., and Simmons, R. L. (1957): Brit. J. Haemat., 3, p. 19.
Joyce, C. R. B., Moore, H., and Weatherall, M. (1954): Brit. J. Pharmacol., 9, p. 463.
Maizels, M. (1949): J. gen. Physiol., 108, p. 247.
Passow, H., and Tillman, K. (1956): Pflugers Archiv. f.g. Physiol., 262, p. 23.
Passow, H., and Schutt, L. (1956): *Ibid.*, 262, p. 193.
Ponder, E. (1948): Haemolysis and Related Phenomena. Churchill, London.
Ponder, E., and Barreto, D. (1957): Blood, 12, p. 1016.
Prankerd, T. A. J. (1955): Brit. J. Haematol., 1, p. 131.
Prankerd, T. A. J., Altman, K. I., and Young, L. E. (1955): J. clin. Invest., 34, p. 1268.
Sandell, E. B. (1950): Colorimetric Determination of Traces of Metals. 2nd edit. Interscience, New York.
Selwyn, J. G., and Dacie, J. V. (1954): Blood, 9, p. 414.
Solomon, A. K. (1952): J. gen. Physiol., 36, p. 57.
Sutherland, D. A., and Eisentraut, A. M. (1956): Blood, 11, p. 1024.
Tosteson, D. C., and Robertson, J. S. (1956): J. cell comp. Physiol., 47, p. 147.
Vincent, P. C., and Blackburn, C. R. B. (1958): Austral. J. exp. Biol., 36, p. 471.
Wilde, W. S. (1955): Ann. Rev. Physiol., 17, p. 17.

MICROBIOLOGICAL ASSAY OF VITAMIN B_{12} CONTENT OF SERUM USING *EUGLENA GRACILIS*

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Hutner and co-workers (1949) showed that vitamin B_{12} was an essential growth factor for the green alga, *Euglena gracilis* var. *bacillaris* and this organism was used by Ross (1950, 1952) to assay the vitamin B_{12} content of human serum.

The specificity of vitamin B_{12} for *Euglena* was investigated by Robbins, Hervey and Stebbins (1950) and Ford (1953) and reviewed by Ford and Hutner (1955). Vitamin B_{12} can be replaced by a few other naturally occurring substances as a growth requirement for *Euglena*, but these have not been found in serum. *Ochromonas malhamensis* is more obligatory in its requirements for vitamin B_{12} than *Euglena gracilis* (Ford, 1953). Mollin and Ross (1955) found comparable values for vitamin B_{12} in serum using both *Euglena gracilis* and *Ochromonas malhamensis*.

Euglena assay has some technical advantages over methods using other organisms. Small quantities only of serum are required for assay and these need no preliminary treatment. The acid pH of the medium allows the serum-medium mixture to be heated to liberate "bound" vitamin B_{12} without precipitation of protein. Both "bound" and "free" values for vitamin B_{12} are readily obtained. *Euglena* compares very favourably with other organisms in sensitivity, being stimulated to growth by as little as 0·5 $\mu\mu$g. vitamin B_{12} per ml. The acid pH of the medium is inhibitory to the growth of most bacteria and contamination of culture tubes seldom occurs.

The medium was not buffered in the method described by Ross (1952). Increasing alkalinity during growth sometimes caused precipitation of serum protein and cloudiness in the culture tube. Results could not be read until 7 days after inoculation. In an effort to overcome these objections, Hutner, Bach and Ross (1956) described a new *Euglena* method using the Z strain (Pringsheim) and a modified medium. The medium contained sugar and was buffered at pH 3·6. The organism grew more vigorously in this medium, and the assay time was shortened to 4 to 5 days. A preliminary comparative study of the two methods was reported by Ross, Hutner and Bach (1957).

Assays have been carried out in this laboratory over the past two years

using both the Z and bacillaris strains of *Euglena*. This paper compares the results obtained with the two methods. Serum vitamin B_{12} values of a large group of healthy subjects are reported also.

METHODS.

Dilutions for the standard growth curve in both methods were made from 1 ml. ampoules of cytamen 20 (Glaxo) bearing the same batch number. An ampoule of this batch was assayed by Dr. Ross to contain 19·85 μg. vitamin B_{12} per ml.

The stock culture was washed before inoculation of test materials in both methods, since Kristensen (1954) showed that this washing made the assay more sensitive. He described a thermolabile factor inhibitory to growth in stock cultures of *Euglena*. This material appears to bind vitamin B_{12} making it microbiologically inactive (Ross, Hutner and Bach, 1957). We have confirmed that a steeper growth curve is obtained using standard amounts of vitamin B_{12} in medium inoculated from washed cultures. Washing was carried out by centrifuging the *Euglena* stock culture in a screw-capped universal container for 3 minutes at 1000 r.p.m. and discarding the supernatant. The organisms were then resuspended by gentle shaking in 20 ml. sterile single strength medium, again centrifuged and the supernatant removed. The inoculum was made up finally by the addition of sterile single strength medium to a volume twice that of the original stock culture.

The standard growth curve was made from tubes containing 0, 1·25, 2·5, 3·75, 5·0, 7·5, 10 and 15 μμg. crystalline vitamin B_{12} per ml. The standard tubes containing 25 and 50 μμg. vitamin B_{12} per ml. described in the method of Ross (1952) were omitted, as growth in these tubes was too dense to record with the absorptiometer used in this laboratory.

Stock cultures of *Euglena* which had grown for 7-14 days were used for inoculation of tubes. Published methods advise using a vigorously growing stock 5-7 days old; the use of young cultures may result in steep growth curves, perhaps indicating carryover of vitamin B_{12} within the organisms. These steep curves introduce an inaccuracy into the assay method. Old stock cultures tend to give more reproducible growth curves, but growth starts more slowly and the tubes may require longer incubation. Growth was read after 6 days incubation with the Z method and 7 days with the bacillaris method.

An internal check on the accuracy of each batch was afforded by the inclusion of a control normal plasma which was kept frozen in suitable aliquots at — 20° C. There has been no deterioration in the B_{12} content of one such plasma kept frozen for more than 12 months.

After inoculation, the tubes were incubated in an air conditioned room in perspex baths 39in. × 20in. × 7in. over four 4 feet "warm white" fluorescent tubes. The water temperature of the baths varied from 25° C. to 29° C. Growth was recorded with a Spectronic 20 absorptiometer at a wavelength of 650A°. The tubes were shaken vigorously on a cork to disperse clumps, allowed to stand 30 minutes to dispel froth, then gently inverted a few times and read immediately. The addition of caprylic alcohol to prevent frothing resulted in the suspension of organisms becoming milky on several occasions and is no longer used.

Scrupulous cleanliness of glassware was essential for reproducible results. After use, all tubes were washed with detergent, rinsed in running tap water, and soaked overnight in chromic acid solution. They were rinsed next in tap water and distilled water and twice autoclaved filled with distilled water at 15 lb. pressure for 15 minutes. They were finally dried in a hot air oven, plugged with cotton wool and sterilized by dry heat.

Both human and quokka sera were studied in this investigation. The quokka is a small marsupial animal with a range of serum vitamin B_{12} content much higher than the human. Quokka sera were used to test the accuracy of the assay methods at high serum vitamin B_{12} concentrations.

RESULTS.

Repeated estimations on the same sample.

A pooled plasma sample stored frozen in aliquots at $-20°$ C. was assayed for total vitamin B_{12} content 76 times with the bacillaris strain and 70 times with the Z strain of *Euglena* in their respective media. Each assay was performed in triplicate at a serum dilution of 1 in 40 and the mean reading used. The results are shown in Table 1. The range of values obtained with this same

TABLE 1.

Range and mean of results of vitamin B_{12} concentration of a pooled plasma sample assayed many times by both bacillaris and Z strains of Euglena gracilis.

Strain	No. of Assays	Vitamin B_{12} ($\mu\mu$g./ml.)			S.E.
		Range	Mean	S.D.	
Bacillaris	76	150-488	305	76	8·7
Z	70	180-380	256	46	5·5

plasma was greater with the bacillaris strain and the mean value and standard deviation were higher than with the Z strain. More reproducible results were obtained using the Z strain. With both methods unaccountable fluctuations occurred from batch to batch. These could be explained partly by fluctuations in the steepness and shape of the standard growth response curves. In general, when a steep standard curve was obtained, the results with sera in that batch tended to be lower than in a batch with a flatter standard curve.

Comparison of results over a wide range of serum vitamin B_{12} concentrations.

Two hundred and fifty-four human and 615 quokka sera were assayed in duplicate batches for total vitamin B_{12} content, once with the bacillaris strain and once with the Z strain of *Euglena* in their respective media. Each assay result was the mean of a triplicate reading. Since these sera covered a wide range of vitamin B_{12} concentration, they were not all assayed at the same serum dilution. However, each individual serum was tested with both strains at the same dilution.

The 254 human sera samples showed vitamin B_{12} concentrations ranging from 20 to 11,000 $\mu\mu$g. ml. Eleven samples gave the same result with both strains. The median value of the remaining 243 samples was 520 $\mu\mu$g./ml. The bacillaris strain recorded the higher value 77 times in the 121 samples with values below the median, and 49 times in the 121 with values above the median.

The 615 quokka samples showed vitamin B_{12} concentrations ranging from 184 to 60,000 $\mu\mu$g./ml. Thirteen samples gave the same result with both strains.

The median value of the remaining 602 samples was 1524 $\mu\mu$g./ml. The bacillaris strain recorded the higher value 185 times in the 301 samples with values below the median and 131 times in the 301 samples with values above the median.

These results indicated that the Z strain assayed lower in the lower ranges and higher in the higher ranges than the bacillaris strain with both human and quokka sera. The χ^2 test was applied to both series and the probability that this distribution was due to chance lies between 0·01 and 0·001.

Normal values for serum vitamin B_{12} concentration in human sera.

Serum samples from 218 adult normal subjects were assayed for total vitamin B_{12} content. To obviate differences in results due to the strain of *Euglena* used for assay, these sera were assayed in duplicate batches, once with the bacillaris strain and once with the Z strain in their respective media. Each assay was performed in triplicate and the mean of the 6 results was recorded as the vitamin B_{12} content of the serum. Two groups of normal sera were assayed. The first consisted of 114 samples supplied by the Red Cross Blood Transfusion Service and taken from consecutive blood donors reporting at the Transfusion Centre. This was a heterogeneous group including both males and females between the ages of 18 and 60. The second group consisted of 104 samples taken from young healthy males. These were National Service trainees who were donating blood for the first time. The results obtained are shown in Table 2. The values ranged from 190 to 875 $\mu\mu$g./ml. with a mean of 456 $\mu\mu$g./ml. (S.D. 133). The values obtained in both groups were very similar.

TABLE 2.

Serum vitamin B_{12} concentrations in normal human subjects.

	No. of subjects	Serum vitamin B_{12} concentration ($\mu\mu$g./ml.)			S.E.
		Range	Mean	S.D.	
Group 1	114	190-875	459	150	14·1
Group 2	104	260-836	453	112	11·0
Total	218	190-875	456	133	9·2

DISCUSSION.

The results show that in the ranges of serum vitamin B_{12} concentration likely to be found in normal human sera, assays with the Z strain of *Euglena gracilis* give lower values than do those with the bacillaris strain. Although the differences noted are unlikely to have any clinical significance, the discrepancy has considerable interest. The Z medium is more balanced than the bacillaris

medium (Hutner, Bach and Ross, 1956) and it is possible that serum would provide a growth stimulus above that due to its vitamin B_{12} content in a deficient medium such as the bacillaris. This would explain the higher values obtained with the bacillaris method in the lower ranges of vitamin B_{12} concentration where serum is used at less dilution. Under these circumstances growth in the control tubes, which contain crystalline vitamin B_{12} but no serum, would not have the benefit of this extra growth stimulus. This effect would become less marked with sera with high vitamin B_{12} concentration because such sera are diluted further. It is probable therefore that the Z method gives a more accurate indication of the true serum vitamin B_{12} concentration.

Ross, Hutner and Bach (1957) reported satisfactory agreement between values for serum vitamin B_{12} concentration obtained with both methods. We have found the range of values obtained by multiple tests on the one plasma sample is large with either method, but more so with the bacillaris strain. The Z technique is therefore more reproducible. These authors assayed a single serum sample 49 times using the Z strain and found a range of values from 190 to 360 $\mu\mu$g./ml. (S.D. 54). This scatter of results is of the same order as we have observed ourselves (Table 1). This degree of variability seems to be a fault inherent in most microbiological assays, but seldom has clinical significance as the normal range is wide and the difference between normal and abnormal usually clear cut.

There is little to choose between the *Euglena* methods using the bacillaris and Z strains from the technical point of view. The assay is shortened by a day or two using the Z strain and the buffered Z medium obviates occasional cloudiness in culture tubes due to protein precipitation following alkali production. *Euglena* growth seems to be more evenly dispersed in tubes using the Z strain. With the bacillaris strain, growth is more prone to occur on the surface of the culture medium and form a ring on the wall of the tube which is difficult to disperse at the time of reading.

The values reported for normal subjects in Table 2 are higher than those reported by most other workers. A comparison of other series of normal results is given by Mollin and Ross (1957) who include their own latest figures. The range of vitamin B_{12} concentrations in 223 sera assayed by Mollin and Ross was from 100 to 900 $\mu\mu$g./ml. with a mean figure of 356 $\mu\mu$g./ml. A mixed group of males and females between the ages of 18 and 60 whose sera was assayed by us gave a closely similar mean result and a similar range to a group of healthy young National Service trainees. This suggests that neither sex nor age in the range stated has any bearing on the serum vitamin B_{12} concentration. In the combined group of 218 sera, the lowest value obtained was 190 $\mu\mu$g./ml. We have observed on several occasions serum vitamin B_{12} concentrations between 100 and 190 $\mu\mu$g./ml. in hospital patients with no evidence of haematological disorders. Perhaps such lower "normal" values in hospital patients are due to

selection from a different class of society. The pensioner group, often with poor dietary habits, and elderly people are likely to be included in any collection of "normal" sera drawn from hospital patients without overt blood disorders. For this reason, our normal range may more accurately represent an optimum than a "normal" for the population as a whole.

SUMMARY.

The vitamin B_{12} content of plasma and serum was measured by microbiological assay using both the Z strain of *Euglena gracilis* in an improved buffered medium and the bacillaris strain in a standard medium. The methods were compared by multiple tests on the same plasma sample and by duplicate assays on many serum samples. Results obtained with the Z strain were more reproducible and probably more accurate than with the bacillaris strain.

Values are given for the range of normal serum vitamin B_{12} concentrations in healthy adults.

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REFERENCES.

Ford, J. E. (1953): Brit. J. Nutrit., 7, p. 299.
Ford, J. E., and Hutner, S. H. (1955): Vitamins and Hormones, 13, p. 101.
Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffman, C. E., Belt, M., Franklin, A. L., and Jukes, T. H. (1949): Proc. Soc. exp. Biol. Med., 70, p. 118.
Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956): J. Protozool., 3, p. 101.
Kristensen, H. P. Ø. (1954): Acta physiol. Scand., 33, p. 232.
Mollin, D. L., and Ross, G. I. M. (1955): Brit. J. Haemat., 1, p. 155.
Mollin, D. L., and Ross, G. I. M. (1957): In "Vitamin B_{12} und Intrinsic Factor", ed H. C. Heinrich (Enke, Stuttgart), p. 413.
Robbins, W. J., Hervey, A., and Stebbins, M. E. (1950): Bull. Torrey Bot. Cl., 77, p. 423.
Ross, G. I. M. (1950): Nature, 166, p. 270.
Ross, G. I. M. (1952): J. clin. Path., 5, p. 250.
Ross, G. I. M., Hutner, S. H., and Bach, M. K. (1957): In "Vitamin B_{12} und Intrinsic Factor", ed. H. C. Heinrich (Enke, Stuttgart), p. 305.

THE CYTOLOGY OF THE PARS DISTALIS OF THE WALLABY PITUITARY

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Studies of the rat pituitary have permitted the establishment of three distinct functional types of "basophils" which have been regarded as FSH-, LH-, and TSH-basophils (Purves and Griesbach, 1951a, 1951b; Farquhar and Rinehart, 1954a, 1954c; Purves and Griesbach, 1954, 1955; Barrnett, Ladman and McAllaster, 1955) and two functional types of acidophils which were thought to produce STH and prolactin (Lacour, 1950; Purves and Griesbach, 1952; Farquhar and Rinehart, 1954). These functional types of acidophils in the rat cannot be satisfactorily demonstrated tinctorially unless a special technique is applied (Dawson, 1954a, 1954c). A possible functional type of chromophobe ("adrenotroph") has been reported for the rat (Farquhar, 1957).

While two tinctorial types of acidophils have been reported for at least seven different orders of mammals (Dawson and Friedgood, 1938; Goldberg and Chaikoff, 1952; Hanström, 1952; Dawson, 1954a, 1954b; Herlant, 1956; Purves and Griesbach, 1957) they have also been found in the anterior lobe of most classes of lower vertebrates (Copeland, 1943; Dawson, 1946; Ortman, 1956).

The staining reactions of anterior pituitary basophil cells of the rat are now quite well known. Aldehyde fuchsin (AF) applied without previous oxydation, stains only the TSH basophils (Halmi, 1950; Purves and Griesbach, 1951b) while there is a positive periodic acid Schiff (PAS) reaction in all types of basophils (Purves and Griesbach, 1951a, 1951b).

A cytological study of the anterior lobe of the dog (Purves and Griesbach, 1957) revealed a PAS reaction in one of the two tinctorial types of acidophils (the epsilon cell of Goldberg and Chaikoff, 1952) and an AF reaction in all three tinctorial types of basophils. Moreover, an intense PAS reaction has been found in one of the two tinctorial types of acidophils in the frog (Ortman, 1956). These studies indicate that cells commonly referred to as "acidophils"

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and "basophils" may differ considerably from their counterparts in different vertebrate pituitaries. More extensive comparative studies of these cells are needed. In this laboratory several years ago one of us (W. E. Griesbach) observed that the pituitaries of marsupials occurring in New Zealand (the scrub wallaby and the brush-tailed opossum) showed two tinctorial types of acidophils and that in the wallaby pituitary one acidophil type had a regionally restricted distribution. Interest was therefore stimulated to study further the staining reactions, distribution, and function of the cells in the pars distalis (p.d.) of the wallaby.

METHODS AND MATERIALS.

Hypophyses of 4 male and 3 female adult scrub wallaby (*Wallabia rufogrisea*) were taken from animals shot in the Hunter Hills, Waimate, New Zealand. These were dissected from the skull immediately after death, placed in formol-saline and later transferred to sublimate-formol for 24 hrs. (9 : 1). They were embedded in Fisher's tissue mat, sectioned at 2·5 micra and the sections stained by the following methods:

(1) Crossmon's (1937) modification of the Mallory stain.
(2) Periodic acid-Schiff (PAS) as used by Purves and Griesbach (1951a), with or without counter-staining by (a) Harris' haematoxylin, (b) fast green (FCF) (0·1 p.c. fast green in 1 p.c. acetic acid).
(3) Aldehyde fuchsin-Bayer (0·5 p.c. in 70 p.c. alcohol with 2 p.c. HCl) was used in preference to the original aldehyde fuchsin stain of Gomori (1950) which was applied in the earlier examinations only.
(4) Toluidine blue (0·5 p.c.). Stained for 20 min. Examined without dehydration.
(5) Methylene blue (Grübler) (0·33 p.c. in the veronal acetate buffer of Michaelis at different pH values). Stained for 10 min. Examined without dehydration.
(6) Methyl green (Grübler) and pyronine G (BDH) made according to Pearse (1953, p. 427), substituting McIlvaine's buffer at pH 5·0. Stained for 20 min. Certain sections were exposed to ribonuclease[2] (1 mg./ml. dist. water) for 1 hr. at 37° C. prior to staining.
(7) Fast green (FCF) (Gurr, London) (0·8 p.c. in McIlvaine's buffer). Stained for 20 min. Rinsed in tap water. Dehydrated in tertiary butyl alcohol.

In order to determine the distribution of the two acidophil cell types in the pars distalis (p.d.) with reference to the exterior of the gland and as a preliminary step in the bioassay of such cells for hormone contents, pituitaries were partially transected from the right and the various planes of transection, from back to front, recorded. The incisions did not extend far beyond the median sagittal plane. In this way, histological examination could determine regional distribution of a certain type of cell with reference to the dividing cut.

The identification of cells, stained, e.g. by the Mallory-Crossmon stain with cells stained by any other stain, was established by repeated photomicrography of selected cell groups before and after counterstaining, or by bleaching and restaining by various staining procedures. Some of the latter procedures are set forth in the footnotes to Table 1.

Preliminary hormone bioassays (S.T.H. and Prolactin) were performed on the two parts of acetone dried, deep frozen anterior lobes of wallaby pituitaries after the glands had been completely transected in the plane described under "Results". The bioassay for Prolactin was based on the pigeon crop sac method, while S.T.H. was assayed by the growth response of the hypophysectomised immature rat.

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[2] Obtained from Worthington Biochemical Laboratory, New Jersey.

RESULTS.

A. *Gross observations.* The pituitary (Fig. 1) is laterally compressed, the dorso-ventral axis deep, and the dorsal aspect oval. The rostral part of the p.d. terminates in a rounded, dorsally pointing projection under the median eminence (M.E.). The pars tuberalis is thin and intimately associated with the M.E. The posterior lobe is enclosed by the p.d. and, therefore, only part of it is visible in Fig. 1. The lower part of the p.d. forms the ventro-lateral lobes which may be seen in Fig. 1.

B. *Cell distribution.* Employing the method of partial transection, mentioned above, we found that if the plane of division was made, as indicated by the line in Fig. 1, under an angle of about 20° from the vertical and passing from the posterior wall of the median eminence to the anterior edge of the ventro-lateral lobe, the p.d. of the pituitary could be cleaved into two parts, the anterior of which contained approximately 95 p.c. of all the acid fuchsin acidophils. We have designated this part as the oral or rostral part. Orange G cells are distributed throughout the p.d. However, orangeophil cells in the rostral part are generally smaller than those in the caudal part. The plane of transection, therefore, also gives a rough separation of the two classes of orange G cells. Basophils and chromophobes are distributed for the most part fairly evenly throughout the p.d.

C. *Staining characteristics of cells in pars distalis.* Most of the staining results are set forth in Table 1 wherein the rostral and caudal regions are defined by the plane of transection given above. It will be observed that although Crossmon's method differentially stains three general types of chromophils (acid fuchsin cell, orange G cell and aniline blue cells), other staining methods employed allow a further separation of these cells (see below).

1. *Acid fuchsin cell* (Figs. 3, 6). This cell is intensely stained by azocarmine (using Koneff's method, 1938), aldehyde fuchsin (Fig. 2) toluidine blue (red or purple), methylene blue (pH 3·9), and pyronine (pH 5·0). This cell is only very slightly coloured by PAS (Fig. 4); consequently it is greyish upon fast green counterstaining. Methylene blue or pyronine stainability is not lessened by RNase treatment. The metachromasia in this cell or in any other chromophil in the p.d. is not alcohol-resistant.

2. *Orange G cells.* In sharp contrast to the previous cell type, these cells are not coloured by aldehyde fuchsin (Fig. 2), but they stain intensely with fast green at pH 5·8. Similar to the previous cell, PAS lends an extremely slight colouration to these cells when in the rostral location. In addition, PAS-positive vesicles may occasionally be observed in the cytoplasm of the orange G cell. Certain of the rostral orange G cells are stained fairly strongly by basic dyes, but this is not the case for the caudally located orange G cells.

Fig. 1. Photographic representation of lateral view of wallaby pituitary. The plane of transection, as drawn by line, separated the pituitary into rostral and caudal parts for bioassay purposes (see text). ME = median eminence.

Figs. 2-6 are photomicrographs of 2·5 μ sections of the wallaby pituitary. Figs. 2-4 (male) demonstrate groups of cells to which our triple strain-sequence was applied, x 900. RA = red acidophil (acid fuchsin cell); OA = orange G cell; B = blue and/or purple basophil; VB = vesiculated basophil.

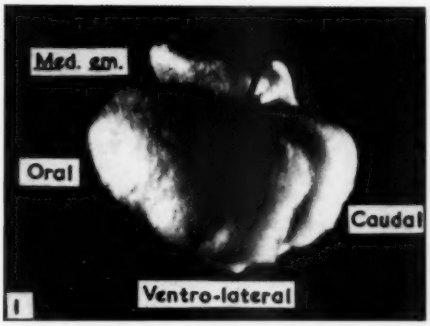
Fig. 2. Rostral part of pars distalis. In the centre and at lower right are numerous AF positive acid fuchsin cells (RA). The orange G cells (OA) at top are unstained. AF-Haematoxylin.

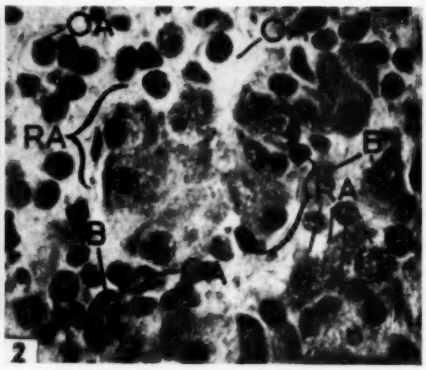
Fig. 3. The cells seen in Fig. 2 have, in addition to the AF, been stained with Crossmon. Due to the fact that acid fuchsin stains the identical cells as the AF, these appear unusually dark. (RA cells.) The orange G cells are also stained. Crossmon.

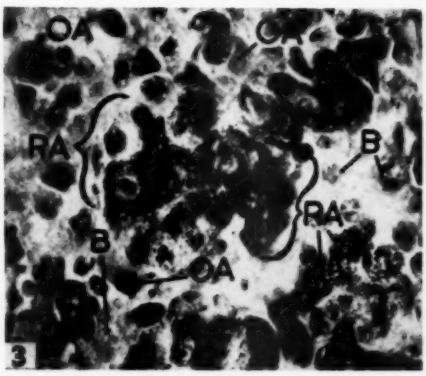
Fig. 4. The cells of Fig. 3 have been bleached and recoloured by PAS. A weak reaction in acid fuchsin cells (RA) may be seen. PAS.

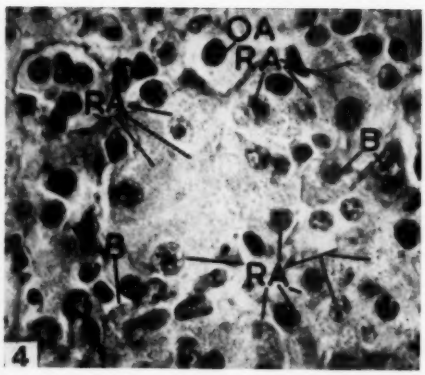
Fig. 5. Rostral Region. Note a very slight PAS reaction in acid fuchsin cell (RA) but an intense PAS reaction in vesicles of vesiculated basophils (VB). PAS.

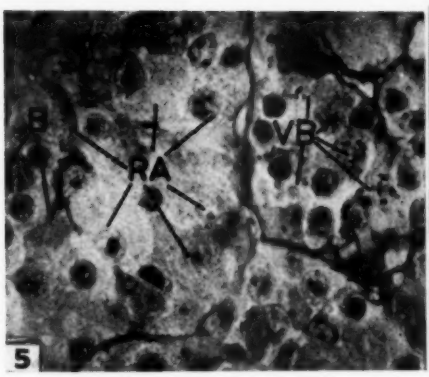
Fig. 6. Same cells as in Fig. 5. Now the acid fuchsin cells (RA) are strongly stained while the vesicles in the basophils (VB) are invisible. Note the orange G cell being partially cupped by an acid fuchsin cell in the upper part of the field. Crossmon.


Med. em.
Oral
Caudal
Ventro-lateral
1


OA
RA
B
A
B
2


OA
RA
B
RA
B
OA
3


OA
RA
RA
B
B
RA
4


B
RA
VB
5

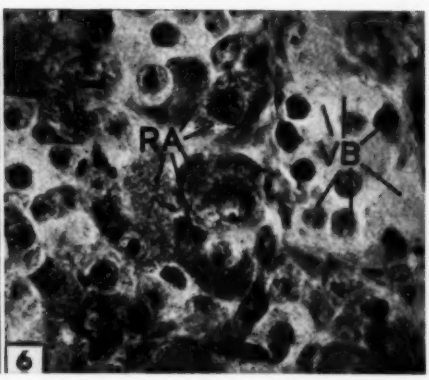

RA
VB
6

TABLE 1.

Cytology properties of chromophils of pars distalis of wallaby.

Region of Pars Distalis	Crossmon Procedure*	Aldehyde Fuchsin†	PAS.†	Toluidine Blue††	Methylene Blue pH 3·9††	Methyl Green-Pyronine pH 5·0††	Methyl Green-Pyronine (after RNase)	Fast Green pH 5·8††
Rostral	Acid Fuchsin cell	+	extremely faint +	metachromatic	+	Pyronine +	Pyronine +	faint +
	Orange G cell Type A Type B	–	extremely faint + + in vesicles	some metachromatic faint orthochromatic or –	+ cells – cells	Pyronine + cells – cells	Pyronine + cells	strong + +
	Cells with blue or/ and purple granules	+ + in vesicles	strong + some cells with + vesicles	metachromatic	+	Pyronine +	reduced Pyronine staining	faint +
	Cells with faint blue granules	–	+					
Caudal	Orange G cell	–	– in granules, + in vesicles in some cells	faint orthochromatic	– or weak +	–	–	strong +
	Cells with blue or/ and purple granules	+	+	metachromatic	+	Pyronine +	reduced Pyronine staining	faint +

* Destain with 70 p.c. ethyl alcohol overnight or by oxidation for 20 seconds in a solution of 0·3 p.c. $KMnO_4$ in 0·3 p.c. H_2SO_4. Sections may be restained with PAS.

† Destain in acid-permanganate as above. Note it is technically possible to carry out a discriminating triple sequence of staining results on any given region as follows: (a) stain with AF, (b) destain with permanganate, (c) PAS reaction, (d) destain with permanganate, (e) Crossmon's.

†† Destain with 90 p.c. alcohol. Sections may be restained with Crossmon's.

(+ = positive staining; – = no staining of cytoplasm.)

3. *Basophils.* With Crossmon's method the basophils show blue or/and purple specific granules. Since there are transitional forms between blue and purple and the intensity of staining may also vary considerably, we have not been able to separate specific basophil cell types by this method of staining. The majority of the basophils stain intensely with PAS (Fig. 4), AF (Fig. 2), and basic dyes. Their basophilia, in contrast to that observed in acidophils, is markedly reduced by previous RN-ase treatment. A minority of basophils are small cells, located in the rostro-ventral lobes and stained only faintly by aniline blue. In certain of these cells vesicles may be demonstrated which are stained by both PAS and AF (Figs. 5 and 6). In others of these cells PAS gives a faint positive, homogeneous reaction, whereas AF does not stain. These latter cells may have a rather dense nucleus.

D. *Bioassay.* Assays for prolactin content of rostral and caudal parts of p.d. so far have not indicated a significant difference between the two parts. Assays for S.T.H. gave a strongly positive result with the tissue of the caudal p.d., which contains concentrated masses of the large orangeophils.

DISCUSSION.

Conventionally, after trichrome procedures such as Crossmon's modification of Mallory's stain, the cells which stain red or orange are termed "acidophils" while those which stain blue or purple are called "basophils". The irrationalities of this nomenclature and the present necessity of its retention have been discussed before by Dempsey and Wislocki (1945), Purves and Griesbach (1951a) and Peterson and Weiss (1955). Aniline blue and PAS staining have been regarded as alternative methods for the demonstration of "basophils" (Purves and Griesbach, 1951a). More recently, cases have been reported where unequivocal PAS reactions are demonstrable in "acidophils" (Ortman, 1956; Purves and Griesbach, 1957). The latter authors (1957) recommended the application of a glycoprotein solubility test before classifying such 'atypical' cells as "acidophil" or "basophil". In the present study our application of the terms "acidophil" and "basophil" to pituitary cells was restricted to the practise of naming cells after their tinctorial reactions with the triple stains (such as Crossmon's).

All the facts available at present from the wallaby pituitary indicate that the chromophils in the pars distalis may be classified tentatively into 7 types:

A. *Acidophils.*

1. *Acid fuchsin cell.* Intensely stained by AF, toluidine blue (metachromatically), methylene blue, pyronine and azocarmine. Faintly stained by fast green and PAS. Basophilia not removable by RN-ase. Rostral distribution.
2. *Rostral orange G cell–type A.* Strongly stained by fast green. Basophilia not removable by RN-ase. Faintly stained by PAS. Negative to AF but positive to several basic dyes (pyronine). Small cells.

3. *Rostral orange G cell–type B.* Strongly stained by fast green. Faintly stained by PAS. Negative to AF and also to basic dyes. Small cells.
4. *Caudal orange G cell.* Strongly stained by fast green. Negative to basic dyes at pH values used, AF, and PAS. Large cell.

B. *Basophils.*

1. *Blue and/or purple basophil.* Positively stained by PAS, AF, toluidine blue (metachromatically), methylene blue, and pyronine. Basophilia is reduced by RN-ase. Faintly stained by fast green. Distributed throughout p.d. These cells are not classifiable into subtypes with the present methods because of presence of cells with transitional tinctorial properties.
2. *Faint blue basophil.* PAS-positive. AF-negative. Small size. Rostral distribution.
3. *Vesiculate basophil.* Stained blue by Crossmon's stain without giving prominence to vesicles in cytoplasm demonstrable by other methods. Vesicles are stained by PAS and AF. Background cytoplasm is weakly PAS-positive. Rostro-ventral distribution.

The pituitary of the short-tailed wallaby (*Setonyx setosus*) has been investigated by Hanström (1953) after application of the kresazan-orange method. He reported the presence of carmine acidophils in the rostral region of the p.d., one type of orange G cell (his "eta" cell) and beta and delta basophils. No sex difference in the basophils was described, but it was noticed that "the eta cells are especially outstanding in the female which also possesses conspicuous small groups of large carminophils in the most rostral region of the lobe".

It seems to be highly probable that the carmine acidophils of Hanström are identical with the acid fuchsin cells of the present paper. But this author has not reported on the uptake, by these cells, of aldehyde fuchsin without preceding permanganate oxydation. Such reaction of an "acidophil" cell was most unexpected and apparently is without parallel. The faint reaction to PAS excludes the possibility that we might deal with a basophil with acidophil inclination as found in the dog and the human pituitary. It is not recommendable therefore to draw conclusions as to the nature or the function of a pituitary cell from its staining qualities only, when glands of different species are examined. The circumstances under which the wallaby pituitaries were collected precluded the application of the glycoprotein solubility test recommended by Purves and Griesbach (1957) for the identification of doubtful pituitary cells.

The bioassay therefore seemed to be the most promising procedure because the regional restriction of the "red acidophil" to the rostral part of the p.d. permitted the comparative assay of two parts of tissue one of which was free of the "red acidophil". Difficulties in collecting the material have so far prevented assays on a large scale. Results of assays carried out up to now did not favour

the hypothesis that the "red acidophil cell" of the wallaby pituitary is associated with prolactin production.

SUMMARY.

The cytology of the pars distalis of the male and the female wallabies was studied with acid and basic dyes (in conjunction with RN-ase), Periodic acid-Schiff (PAS) and aldehyde fuchsin (AF).

The chromophils of the pars distalis may be tentatively classified into 4 types of "acidophils" and 3 types of "basophils".

The acid fuchsin cell (= carmine cell) has the same distribution (rostral) in the pars distalis of either sex. A method is presented whereby it is possible to cut the pituitary in order to separate the acid fuchsin cell from the caudal orange G cell.

The acid fuchsin cell of the wallaby is unique in its cytologic properties. Though it has been tentatively classified as an apparent "acidophil", it stains vigorously with aldehyde fuchsin and only faintly with PAS.

Prolactin bioassays of wallaby pituitaries, cut in the prescribed manner, indicated that this hormone could not be associated with the acid fuchsin cell.

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REFERENCES.

Barrnett, R. J., Ladman, A. J., and McAllaster, N. J. (1955): J. Histoch., Cytoch., 3, p. 391.
Copeland, D. E. (1943): J. Morphol., 72, p. 379.
Crossmon, G. (1937): Anat. Rec., 69, p. 33.
Dawson, A. B. (1946): Amer. J. Anat., 78, p. 347.
Dawson, A. B. (1954a): Anat. Rec., 120, p. 810, Abstr. 223.
Dawson, A. B. (1954b): *Ibid.*, 120, p. 810, Abstr. 224.
Dawson, A. B., and Friedgood, H. B. (1938): Stain Technol., 13, p. 17.
Dempsey, E. W., and Wislocki, G. B. (1945): Amer. J. Anat., 76, p. 277.
Farquhar, M. G. (1957): Anat. Rec., 127, p. 291, Abstr. 64.
Farquhar, M. G., and Rinehart, J. F. (1954a): Endocrinology, 54, p. 516.
Farquhar, M. G., and Rinehart, J. F. (1954b): *Ibid.*, 55, p. 857.
Goldberg, R. C., and Chaikoff, I. L. (1952): Anat. Rec., 112, p. 265.
Gomori, G. (1950): Amer. J. clin. Path., 20, p. 665.
Halmi, N. S. (1950): Endocrinology, 47, p. 289.
Hanström, B. (1952): Arkiv. f. Zoologie, 4, p. 187.
Hanström, B. (1953): *Ibid.*, 6, p. 98.
Herlant, M. (1956): Arch. Biol., 67, p. 89.
Koneff, A. A. (1938): Stain Technol., 13, p. 49.
Lacour, F. (1950): C.R. Soc. Biol., Paris, 144, p. 248.
Ortman, R. (1956): J. Histochem. and Cytochem., 4, p. 471.
Pearse, A. G. E. (1953): Histochemistry. Theoretical and Applied, Little and Brown and Co., Boston.

Peterson, R. R., and Weiss, J. (1955): Endocrinology, 57, p. 96.
Purves, H. D., and Griesbach, W. E. (1951a): *Ibid.*, 49, p. 244.
Purves, H. D., and Griesbach, W. E. (1951b): *Ibid.*, 49, p. 652.
Purves, H. D., and Griesbach, W. E. (1952): Proc. Univ. Otago med. Sch., 30, p. 27.
Purves, H. D., and Griesbach, W. E. (1954): Endocrinology, 55, p. 785.
Purves, H. D., and Griesbach, W. E. (1955): *Ibid.*, 56, p. 374.
Purves, H. D., and Griesbach, W. E. (1957): J. Endocrin., 14, p. 361.